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Selective Depletion of Alloreactive T Cells to Reduce Graft-Versus-Host Disease and Enhance Immune Reconstitution post Allogeneic Haematopoietic Stem Cell Transplantation

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A Thesis Submitted to the University of London for the Degree of Doctor of Philosophy in the Faculty of Medicine

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For Beth

Abstract

Non-selective T cell depletion reduces the incidence of severe graft-versus-host disease after allogeneic haematopoietic stem cell transplantation but the cost is delayed and disordered antigen-specific immune reconstitution and increased infection. A method of selective depletion of alloreactive donor T cells expressing the activation marker CD69 after co-culture with recipient stimulator cells in a mixed lymphocyte reaction has previously been shown to reduce alloreactivity, whilst retaining third party responses *in vitro* and in a mismatched murine model led to donor T cell engraftment with a virtual absence of GvHD and significantly increased survival.

We have further developed this technique by comparing two methods of potentiating allostimulation in the HLA-matched setting. Cytokine pre-treated recipient stimulator cells led to successful allostimulation of a minority of HLA-matched responder cells whereas the strategy of pre-treatment of recipient stimulator cells with OKT3 led to effective allostimulation in all pairs tested and led to more efficient selective abrogation of alloresponses after depletion of responder alloreactive cells.

The retention of donor antiviral T cell frequencies were compared after selective HLA matched allodepletion using both techniques of potentiating allostimulation. Using both techniques the majority of CMV-specific T cells (quantified by by HLA Class I tetramer assay and IFN-γ ELISpot) and EBV-specific T cells (quantified by IFN-γ ELISpot) were retained in the selectively allodepleted T cell pool. Preservation of antiviral CTLs in selectively allodepleted stem cell grafts would lead to improved antiviral immunity post transplant.

The phenotypic characteristics of the alloreactive and non-alloreactive T cells within the donor pool were examined. $CD69^+$ alloreactive T cells were found to consist of both naïve and memory T cells and to exhibit significant skewing of TCR Vβ sub-family distribution in both the HLA-mismatched and HLA-matched setting. The technique of selective allodepletion based on CD69 expression was found to retain functional $CD4^+CD25^+$ T regulatory cells The retention of immunosuppressive $CD4^+CD25^+$ T regulatory cells could lead to more ordered immune reconstitution and further suppress alloreactive responses post

transplant. Direct stimulation of donor T cells with CMV peptide led to up regulation of CD69 on CMV-specific T cells and these cells exhibited TCR Vβ sub-family overuse consistent with previously published data. Homology of TCR Vβ sub-family overuse in HLA A*0201^{+} donor T cells following CMV peptide stimulation and HLA-matched allostimulation was demonstrated in some individuals suggesting supporting the existence of donor T cells possessing TCRs with affinity for CMV and minor histocompatability antigens. Sequential selective allodepletion and CMV antigen stimulation of donor T cells might lead to production of CMV-specific non-alloreactive donor T cell pools suitable for use as adoptive immunotherapy post-allogeneic haematopoietic stem cell transplantation.

The techniques for allostimulation and selective allodepletion at a clinical scale and under sterile conditions have been developed in order to test the safety and the efficacy of this technique in a clinical pilot study of HLA-matched sibling donor allogeneic haematopoietic stem cell transplantation in adults with acute myeloid leukaemia.

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Abbreviations

AHSCT	Allogeneic Haematopoietic Stem Cell Transplantation
AICD	Activation-Induced Cell Death
APCell	Antigen Presenting Cell
APC	Allophycocyanin
ASBMT	American Society of Blood and Marrow Transplantation
BSA	Bovine Serum Albumin
CAI	Calcium Ionophore
CDR3	3rd Complimentarity Determining Region
CE	Conformité Européene
CFSE	Carboxyfluorescein Diacetate Succinimidyl Ester
CML	Chronic Myeloid Leukaemia
CMV	Cytomegalovirus
cpm	counts per minute
CTL	Cytotoxic T lymphocyte
cTLP	Cytotoxic T Lymphocyte Precursor
CV	Co-efficient of Variation
DC	Dendritic Cell
DLI	Donor Lymphocyte Infusions
DMSO	Dimethylsulphoxide
DoH	Department of Health
EBV	Epstein-Barr Virus
ECTD	European Clinical Trials Directive
EDTA	Ethylene Diamine Tetra-acetic Acid
ELISA	Enzyme Linked Immunosorbent Assay
ELISpot	Enzyme-linked Immunospot
FACT	Foundation for the Accreditation of Cellular Therapy
FCS	Foetal Calf Serum
FITC	Fluoroscene Isothiocyanate
FSC	Forward Scatter
GAM	Goat Anti-Mouse
GLP	Good Laboratory Practice

GMP	**Good Manufacturing Practice**
GvHD	**Graft-versus-Host Disease**
GvL	**Graft-versus-Leukaemia**
HAS	**Human Albumin Solution**
HBSS	**Hanks' Balanced Salt Solution**
HDM	**High Density Microbeads**
HEV	**High Endothelial Venules**
HLA	**Human Leucocyte Antigen**
hTLP	**Helper T Lymphocyte Precursor**
IFN-γ	**Interferon Gamma**
IL	**Interleukin**
ISCT	**International Society for Cellular Therapy**
ITAM	**Immunoreceptor Tyrosine-based Activation Motif**
JACIE	**Joint Accreditation Committee in Europe**
KD	**Kilodalton**
LB	**Leukaemic Blast**
LCL	**Lymphoblastoid Cell Line**
LPS	**Lipopolysaccharide**
MCA	**Medicines Control Agency**
mHag	**Minor Histocompatability Antigen**
MHC	**Major Histocompatability Complex**
MHRA	**Medicines and Healthcare Products Regulatory Authority**
MLR	**Mixed Lymphocyte Reaction**
MMF	**Mycophenolate Mofetil**
MPC	**Magnetic Particle Concentrator**
MSE	**MHC-stabilization Efficiency**
NK	**Natural Killer**
NOD/SCID	**Non-obese Diabetic/Severe Combined Immunodeficient**
OVA	**I-A (d)-restricted Ovalbumin**
PBMC	**Peripheral Blood Mononuclear Cell**
PBS	**Phosphate-Buffered Saline**
PBSC	**Peripheral Blood Stem Cell**
PCR	**Polymerase Chain Reaction**

PDP	Photodynamic Purging
PE	Phycoerythrin
PerCP	Peridinin Chlorophyll Protein
PHA	Phytohaemagglutinin
PI	Propidium Iodide
PMA	Pokeweed Mitogen
PTLD	Post-Transplant Lymphoproliferative Disorder
PVDF	Polyvinylidene Difluoride
RPMI	Roswell Park Memorial Institute
RRI	Relative Response Index
S: R	Stimulator: Responder
SAM	Sheep Anti-Mouse
SOP	Standard Operating Procedure
SSC	Side Scatter
SSOP	Sequence-Specific Oligo Primer
TAP	Transporter-associated Protein
T_{CM}	T Central Memory
TCR	T Cell Receptor
TDC	Terminally Differentiated Cells
T_{EM}	T Effector Memory
T_H1	T Helper 1
T_H2	T Helper 2
TNF-a	Tumour Necrosis Factor Alpha
TSE	Transmissible Spongiform Encephalopathy
vs	versus
WinMDI	Windows Multiple Document Interface for Flow Cytometry

List of Reagent Suppliers

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WinMDI	www.microsoft.com

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Chapter 1 Introduction

1.1 Haematopoiesis

In 1909 Alexander Maximov first postulated that among the lymphocytes in the peripheral blood, a population of common stem cells (*gemeinsame stammzellen*) circulated that had the ability to differentiate into all lineages of haematopoietic cells.[Maximow A. 19090] Human blood cells are derived from such pluripotent stem cells that are capable of self-renewal and reside within the adult bone marrow.[Payne and Crooks 2002] Haematopoiesis, the division of haematopoietic stem cells and their subsequent maturation and differentiation into specialised cell lineages within the bone marrow, occurs under the influence of haematopoietic growth factors and adjacent bone marrow stromal cells.[Cantor and Orkin 2001] The production of functional blood cells by the bone marrow can be impaired or totally destroyed by pathological processes affecting the bone marrow stroma or stem cells. Such processes include leukaemic transformation of haematopoietic cells and infiltration of the bone marrow by malignant non-haematological tissues.

1.2 Allogeneic Haematopoietic Stem Cell Transplantation

The therapeutic benefit of administration of saline extracts of bone marrow to patients with anaemia was demonstrated as early as the 1920s. [Leake CD 1929] Allogeneic (Greek, from allos, *other*; and gene, *race*) transplantation of haematopoietic stem cells (in the form of spleen cells) was first shown to lead to regeneration of peripheral blood counts in lethally irradiated mice in 1951.[Jacobson *et al.* 1951] In the same year Lorenz et al showed that lethally irradiated mice and guinea pigs could be protected by the intravenous injection of bone marrow from litter mates.[Lorenz E *et al.* 1951] Transplantation of cellular material (as opposed to humoral factors) in such mice led to the regeneration of donor-derived blood cells.[Ford *et al.* 1956] Subsequent experiments in lethally irradiated mice examined bone marrow repopulation by spleen cells and led to the concept of 'multipotential colony-forming units'. Extension of this work with dosing studies showed that repopulation of mouse bone marrow could be achieved with the administration of a single cell, leading

to the concept of the self-renewing pluripotent stem cell.[Till JE and McCulloch EA 1961]

The capacity for 'self-renewal' of haematopoietic stem cells was exploited in the first allogeneic bone marrow transplants in humans. There are many case reports of such procedures undertaken in the 1950s. Mathe reported good evidence of (albeit transient) allogeneic engraftment (as evidenced by red cell antigens) in a group of scientists who had been accidentally exposed to sub-lethal doses of ionising irradiation and subsequently given multiple allogeneic marrow infusions from family members, autologous marrow regeneration eventually occurring.[Mathe G *et al.* 1959] Allogeneic haematopoietic stem cell transplantation (AHSCT) was subsequently undertaken as a treatment for acute leukaemia and aplastic anaemia. The determination of radiation doses needed for recipient marrow ablation, the practical procedure of marrow harvesting and development of supportive treatment of bone marrow transplant recipients were undertaken by E. Donnell Thomas in the 1960s, (now widely recognised as the forefather of modern AHSCT), for which he received the Nobel Prize for Medicine.

AHSCT was first performed in patients with acute leukaemia and aplastic anaemia whose life expectancy would otherwise have been very limited.[Thomas *et al.* 1971] [Pillow *et al.* 1966]The use of AHSCT was subsequently extended to patients with chronic myeloid leukaemia, and malignant lymphomas.[Mackinnon and Goldman 1988;Thomas, *et al.* 1971] More recently the spectrum of diseases successfully cured by AHSCT has widened further, including chronic non-malignant haematological conditions (structural haemoglobinopathies, thalassaemias), congenital and inherited immune deficiencies and some severe metabolic disorders.[Bernaudin *et al.* 1993;Hirschhorn 1980;Lucarelli *et al.* 1984;Yamamura *et al.* 1972] With the establishment of international unrelated donor volunteer bone marrow transplant registries and the advent of molecular HLA typing techniques, allogeneic transplantation of haematopoietic stem cells from matched unrelated donors has become possible. [Davies *et al.* 2000;Petersdorf *et al.* 1998;Shaw *et al.* 2001]

Cord blood is rich in haematopoietic stem cells and many cord blood banks have been established in the past 20 years, providing an additional source of donor cells available for AHSCT, particularly for paediatric recipients.[Almici *et*

al. 1995;Gluckman *et al.* 1992;Laughlin 2001] Recent advances in graft T cell depletion techniques (*vide infra*) have allowed AHSCT between haplotypically-matched individuals.[Aversa *et al.* 1998] Thus both the spectrum of diseases successfully treated with AHSCT and the availability of a donor to an individual requiring an AHSCT have greatly increased in recent years.

1.3 Alloreactivity

Alloreactivity is the term given to the immune system's recognition of non-self in the setting of allogeneic transplantation and is the immunological process that underlies both graft rejection and in AHSCT, Graft-versus-Host Disease (GvHD).

The human immune system has evolved to recognise and destroy foreign ('non-self') antigens whilst acquiring tolerance to components of the body's own cells -'self ' molecules. Since the advent of allogeneic transplantation the understanding of this recognition of self and non-self has assumed much greater importance clinically. A complex of closely linked and highly polymorphic alleles was first recognized as the major histocompatability complex (MHC) in mice.[Counce S and Smith P 1956] These alleles coded for molecules involved in the recognition of self and non-self, and the human counterpart (residing on chromosome 6) was identified as the human leukocyte antigen (HLA) system in 1965.[Dausett J and Rapaport FT 1965] It was soon realised that HLA discrepancy between donor and recipient was the major determinant of both graft rejection and also the already recognised *secondary syndrome* of GvHD. Shortly after HLA typing techniques became available the first HLA-matched sibling AHSCT was performed in 1968.[De Koning *et al.* 1969] Globally, fewer than 25% of patients requiring AHSCT have a fully HLA-matched sibling donor but many can be found a fully HLA-matched unrelated donor via international bone marrow donor registries.[Cleaver 1993] High resolution molecular HLA typing at allelic level has revealed a much higher level of polymorphism in HLA molecules than had previously been identified using serological HLA typing techniques and molecular HLA typing has become a pre-requisite for selection of HLA-matched unrelated donors. [Little and Madrigal 1999]

In HLA-mismatched unrelated donor AHSCT the incidence of both graft rejection and GvHD are inversely correlated to the degree of closeness of HLA matching between donor and recipient and this translates into an increased survival benefit for those transplanted with more-closely matched grafts. Conflicting evidence has been published regarding which mismatched HLA locus is most likely to result in GvHD.[Petersdorf *et al* 1998;Sasazuki *et al.* 1998] Although AHSCT from related donors with one full haplotypical mismatch (typically a parent) can now undertaken with relative safety, the incidence and severity of GvHD is unacceptably high unless the graft is profoundly T cell depleted and contains a very high dose of $CD34^+$ cells to ensure engraftment. Such graft manipulation results in reduced quantitative and qualitative immune reconstitution in the host post-transplant and an increase in post transplant mortality and morbidity from infections.[Aversa *et al* 1998;Keever *et al.* 1989] The use of unmanipulated grafts in AHSCT from HLA genotypically identical siblings still leads to clinically significant GvHD in 20-30% of recipients. [Chao and Schlegel 1995]

The first of a novel system of human antigens subsequently implicated in the development of GvHD in recipients of HLA-matched AHSCTs was first described over 2 decades ago, the H-Y antigen.[Goulmy *et al.* 1976] This antigen has been joined by many others, the genes for which are located on sex chromosomes or autosomes, and are now collectively termed the minor histocompatability antigens (mHags). These mHags are naturally processed peptide fragments originating from intracellular proteins that associate with HLA molecules.[Goulmy 1997]

Donor T cells recognise mHags in an HLA-restricted manner. Some mHags have been shown to have heterogeneous tissue distribution.[Warren *et al.* 1998] After AHSCT, those HLA molecules and mHags that are disparate between donor and recipient collectively form the *alloantigen* repertoire. There is frequently disparity between donor and recipient mHags in the HLA-matched sibling setting. mHag-specific cytotoxic T lymphocytes can be detected in AHSCT recipients with GvHD by the use of tetrameric HLA class I-mHag peptide complexes, and disparity for some mHags has been shown retrospectively to be associated with an increased risk of acute GvHD after HLA identical sibling AHSCT.[Gallardo *et al.* 2001;Mutis *et al.* 1999a] Conversely in

the syngeneic setting where there is complete genetic identity of donor and recipient at all HLA and most mHag loci, GvHD is rarely seen.

Allogeneic proteins are processed into antigenic peptides by antigen presenting cells (APCells). Peptide loading of MHC Class I molecules occurs in the endoplasmic reticulum and in cytosolic vesicles in the case of MHC Class II molecules, which are the transported to the cell surface. Specialised ('professional') APCells include dendritic cells and are distributed in high density in the areas of the body that interface with the external environment (skin, mucosa) and frequently encounter antigen. They take up antigen in these sites by endocytosis and migrate to the lymph nodes where they present antigen to T cells. Immature dendritic cells express high levels of surface molecules, which aid the endocytosis of antigen, whereas mature dendritic cells express very high levels of MHC Class II molecules and co-stimulatory molecules to facilitate efficient antigen presentation. Macrophages, activated B cells and human T cells (under some circumstances) are also able to present antigens. [Arnold *et al.* 1997;Mannie *et al.* 1998;Mannie and Walker 2001;Pichler and Wyss-Coray 1994;Tsang *et al.* 2003]

Classical human MHC Class I molecules (HLA A, B and C) are membrane bound proteins expressed almost universally on nucleated cells. The three extracellular domains of the MHC Class I molecule are associated with β2-microglobulin. Antigen in the form of peptide is bound between two of the extracellular domains. Antigens that bind to MHC Class I molecules are principally endogenous or derived from virus after intracellular processing. Human MHC Class II molecules (HLA-DP, HLA-DQ and HLA-DR) consist of two extracellular polypeptides, each of which has two domains, and antigen binds between the two extracellular polypeptides and is usually exogenous in nature.[Bryant *et al.* 2002;Moron *et al.* 2004]

T cells bear surface membrane receptors belonging to the immunoglobulin superfamily. 90% of mature T cells express T cell receptors (TCRs) consisting of two α chains and two β chains. Each α and each β chain consists of a constant region and a highly polymorphic variable region. It is the latter that binds to processed antigenic peptide when presented to the TCR whilst in association with an MHC molecule. The TCR also consists of five associated polypeptides: CD3-ε, CD3-δ, CD3-γ and usually a ζζ homodimer. These

associated chains are involved with signal transduction and activation of the T cell, and are identical on all T cells.

Peptide antigens sited in their MHC molecules make contact with T cells; every antigen/MHC molecule will only interact with high affinity with T cells bearing the corresponding specific T cell receptor.[Davis and Bjorkman 1988] $CD4^+$ T cells interact predominantly with peptide antigen presented in context of an MHC Class II molecule whereas $CD8^+$ T cells interact predominantly with peptide antigen presented in context of an MHC Class I molecule. However, dual HLA class I and class II restricted recognition of alloreactive T lymphocytes mediated by a single T cell receptor complex has recently been described. The alloreactive human $CD8^+$ T cell clone MBM15 was found to exhibit dual specificity recognizing both an antigen in the context of the HLA Class I A*0201 molecule and an antigen in the context of the HLA Class II DR1.[Heemskerk *et al.* 2001]

Other molecules are involved in strengthening the interaction between the APCell and the T cell (the 'immunological synapse'). Both the CD4 and the CD8 receptor increase the affinity of this association. Adhesion molecules stabilise the peptide antigen/MHC molecule interaction with the TCR. T cell activation stimulated by the contact between peptide antigen/MHC molecules and the corresponding TCR can be augmented by interaction between complementary receptor pairs on the APCell and T cell. The most important of these are the CD28 receptor, expressed on many T Cells, which interacts with the B7.1 (CD80) and B7.2 (CD86) molecules on the APCs. Lack of additional CD28 co-stimulation during antigen presentation to a T cell can lead to specific hyporesponsiveness of that T cell, a state called T cell anergy. Inhibitory co-stimulatory molecules also exist on APCells, the best-described being CTLA-4 (CD152) which is structurally related to CD28, binds CD80 and CD86 but delivers an inhibitory signal to the T cell.[Allison 1994;Mescher 1995]

The high affinity stabilised interaction between antigen (presented in the context of MHC molecule) and the TCR triggers a cascade of intracellular events that lead to T cell activation. The intracellular portion of the TCR complex contains an invariant region, the immunoreceptor tyrosine-based activation motif (ITAM). The TCR complex and CD4 (or CD8) co-receptors are associated via ITAMs with the Src-family protein kinases Fyn and Lck respectively. Upon formation of the immunological synapse Fyn and Lck become activated and activate the

tyrosine kinase ZAP-70, which in turn activates phospholipase C-γ. Three important intracellular pathways are then initiated. Firstly, the transcription factor NF-κB is activated, secondly calcineurin activates the transcription factor NFAT and thirdly Ras activates a MAP kinase cascade that in turn activates Fos, a component of the AP-1 transcription factor. These three transcription factors act to induce specific gene transcription. Two important genes that demonstrate marked upregulation of transcription upon activation of T cells are the Interleukin 2 (IL-2) and the IL-2 receptor genes. Increased production of IL-2 leads to increased T cell proliferation via the autocrine positive feedback loop mediated by increased cell surface expression of the IL-2 receptor.[Carpino *et al.* 2004;Denny *et al.* 2000;Judd and Koretzky 2000]

The physiological process of T cell development and maturation occurs in the human thymus. Here the TCR genes are randomly rearranged in every T cell to generate a highly diverse repertoire of T cells.[Zuniga-Pflucker 2004] After this has occurred dendritic cells within the thymic microenvironment present self-antigens as MHC bound peptides and all T cells whose receptors recognise self antigens are deleted, which results in immunological tolerance to self.[Nossal 1994] Negative selection of B cells also occurs. These processes are not completely effective and both T and B cells with affinity for self-antigens may escape the thymus. These cells are normally inhibited by clonal anergy or by T regulatory cells and lose their abilities as effectors of an immune response. It has been proposed that the loss of function of T regulatory cells leads to increased activity of autoreactive T and B cells and that this may lead to autoimmune disease.[Saoudi *et al.* 1996]

The molecular basis of alloreactivity remains to be fully defined. Between 0.1% and 10% of an individual's T cell repertoire react with alloantigens,[Ashwell *et al.* 1986a;Lindahl and Wilson 1977a;Lindahl and Wilson 1977b;Matesic *et al.* 1998;Suchin *et al.* 2001] compared with a frequency of <1/100000 for nominal peptide antigens.[Karulin *et al.* 2000]

Initial hypotheses of the mechanisms underlying alloreactivity relied on the principle of cross-reactivity of host TCRs for allogeneic MHC/peptide complexes in a *peptide-dominant* fashion.[Lechler and Batchelor 1982] Alternatively there is some evidence that the allogeneic MHC molecule itself may be the conformational structure to which alloreactive T cells respond, independent of the bound peptide (*MHC dominance*).[Liu *et al.* 1993] As understanding of the

molecular interaction between MHC molecules, the peptides they bind and the TCR with which they interact has deepened, several mechanisms have been suggested to explain recognition of foreign MHC molecules. Fortuitous contacts between the TCR and polymorphic helical residues on the allogeneic MHC not present on self-MHC may augment TCR contacts. Since such allogeneic MHC specificities were not deleted by negative selection in the thymus due to their absence, the TCR–allogeneic MHC affinity may be high and T cell activation robust.[Sykulev *et al.* 1994] Furthermore, these additional TCR–MHC contacts may relax specificity requirements for peptide residues, thereby allowing more T cells to respond to allogeneic stimulator cells expressing a range of bound peptides.[Daniel *et al.* 1998] Second, as many polymorphic residues of the MHC antigen-presenting platform are not exposed to the TCR but do contribute to peptide binding specificity, different pools of peptides are bound by different MHC alleles. These variations in peptides may affect thymic selection processes differentially as well.[Kranz 2000] Third, differences in conformation of the same bound peptide in the groove of two different MHC alleles may result in differences of TCR recognition.[Hennecke and Wiley 2002] Thus the alloantigen should be viewed as a combination of foreign MHC and the peptides bound therein.

Two pathways have been described by which the alloantigen complex may be presented, first defined by Lechler and Bachelor.[Lechler and Batchelor 1982] Presentation by donor APCells is termed direct alloantigen presentation. Indirect alloantigen presentation can also occur when foreign MHC molecules are processed and the derived peptides presented by host APCells. Although the relative importance of direct and indirect alloantigen presentation in the clinical AHSCT setting has yet to be determined, it has been shown in vitro that the frequency of human T cells engaged in the indirect pathway of allorecognition is about 100-fold lower than that of T cells participating in the direct recognition of native HLA-DR antigen.[Liu *et al* 1993] A cartoon of these two pathways of alloantigen presentation is shown in Figure 1.1.

Allogenicity still occurs between genotypically HLA identical siblings and this is due to differences in mHags. Recipient mismatching for the mHag HA-1 has been associated with acute GVHD after HLA identical sibling bone marrow transplantation.[Tseng *et al.* 1999]

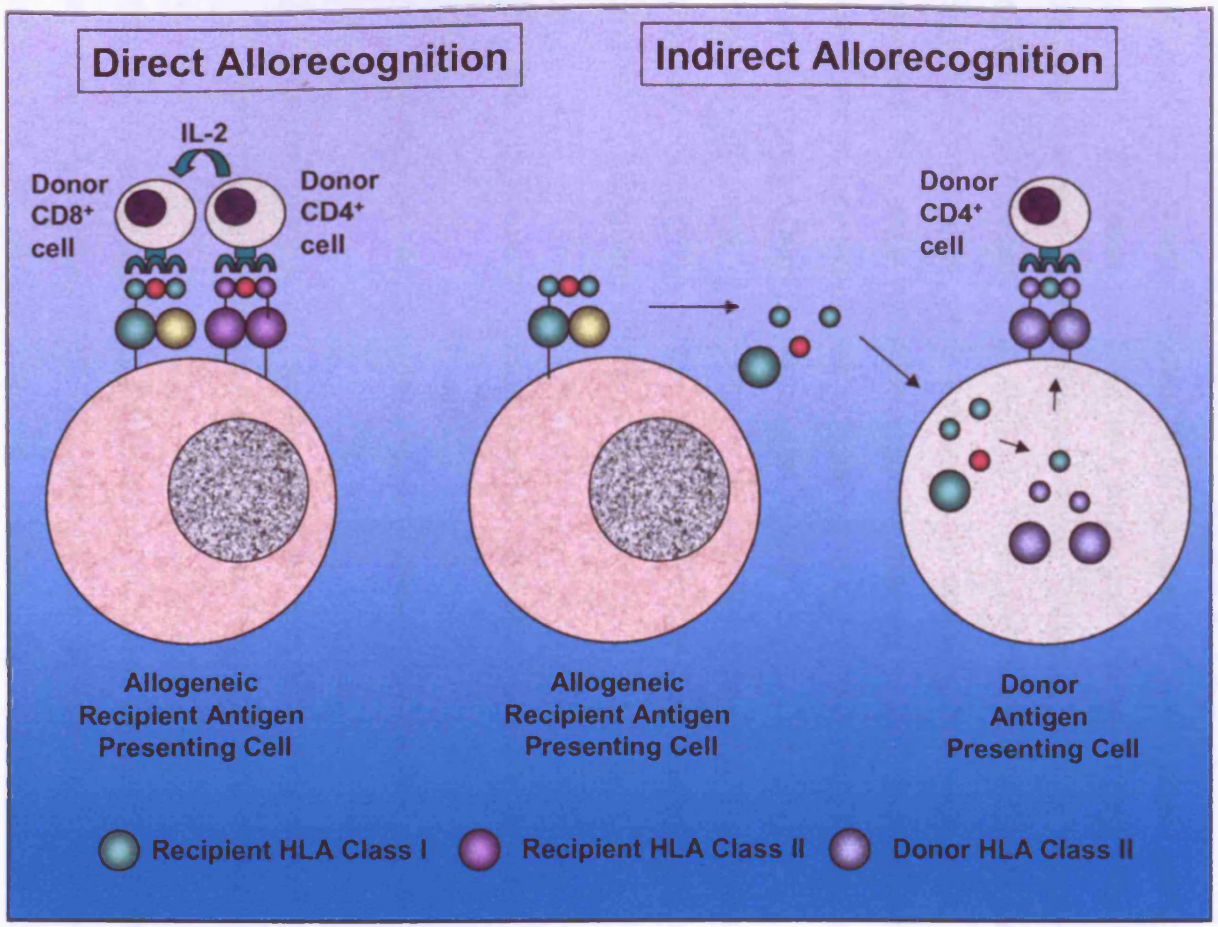

Figure 1.1 Direct and indirect pathways of allorecognition (as defined by Lechler and Bachelor).[Lechler and Batchelor 1982]

1.4. Graft-versus-Host Disease

The major barrier to solid organ allogeneic transplantation in humans is host rejection of the transplanted tissue and long-term host immunosuppression is required to overcome this obstacle. Transplantation of allogeneic haematopoietic stem cells differs from solid organ transplantation in that the transplanted tissue itself constitutes a functionally immunocompetent organ. As a result alloreactivity in AHSCT is *bi-directional.* Allogeneic haematopoietic stem cell grafts are not only capable of being rejected by the host (graft rejection) but are able to recognise the host as foreign and attempt to reject the host tissues. This phenomenon is Graft-versus-Host Disease, and is a major cause of morbidity and mortality post AHSCT. The phenomenon of GvHD was first noted when irradiated mice were infused with allogeneic spleen cells. These mice recovered from their radiation-induced marrow aplasia but subsequently died from a 'secondary syndrome' consisting of diarrhoea, weight loss, skin changes and liver disease.[Van Bekkum *et al.* 1967]
Initial experimental observations of van Bekkum and De Vries led to the theoretical formulation of the requirements for the development of GvHD by Billingham, [Billingham 1966] namely that;

(a) the allogeneic graft must contain immunologically competent cells,

(b) the recipient must be incapable of destroying the graft cells

(c) the recipient must express tissue antigens (now known to be HLA molecules and/or mHags) that are not present in the donor.

Billingham's requirements are fulfilled in all clinical situations where GvHD may be observed, namely AHSCT and/or the subsequent infusion of donor lymphocytes to control disease relapse, transplantation of small bowel containing functional lymphoid tissue, and transfusion of unirradiated blood to immunocompromised patients.
Acute GvHD can occur within days after AHSCT (and by definition occurs within the first 100 days). Host organs affected are those rich in APCells- commonly the skin, gut and the liver. The classical three-step model of the

immunopathogenesis of GvHD was first described by Ferrara.[Ferrara *et al.* 1985] In step one, the recipient conditioning regimen simultaneously damages and activates host tissues, leading to secretion of pro-inflammatory cytokines such as tumour necrosis factor-alpha (TNF-α) and IL-1, and upregulation of adhesion molecules and MHC/antigen complexes on host tissues.[Leeuwenberg *et al.* 1988;Norton and Sloane 1991;Xun *et al.* 1994] This amplifies antigen presentation to allogeneic donor T cells. In step two, donor T cells, activated by host alloantigens proliferate and secrete a variety of cytokines. Donor $CD4^+$ T cells secrete T helper 1 (Th1)-type cytokines such as IL-2 and interferon-gamma (IFN-γ) critical for the development of acute GVHD.[Via and Finkelman 1993] Th2-type cytokines (e.g. IL-4) are also released but the donor $CD4^+$ T cell cytokine profile is deviated towards Th1-type cytokine release. The third step represents the effector arm of the process. Damage to host tissues occurs in the third step and may be the result of the direct effects of cytokines or cytolytic cell pathways.

Mononuclear phagocytes, primed during step two by Th1-type cytokines receive a second trigger signal from lipopolysaccharide (LPS) released from the gut mucosa during step one. LPS may also stimulate keratinocytes and macrophages in the skin to produce inflammatory cytokines.[McKenzie and Sauder 1990] Release of TNF-α and nitrous oxide can also cause direct host tissue damage in the gut. Cytolytic pathways are at least as important as cytokines in mediating host tissue damage in the third step of the pathogenesis of GvHD. Donor $CD8^+$ cytotoxic lymphocytes cause host cell death by both the direct cytolytic damage that results from perforin and granzyme release and by the Fas-Fas Ligand triggering of target cell apoptosis.[Graubert *et al.* 1997;Via *et al.* 1996] The latter mechanism is probably the most important in the pathogenesis of hepatic GvHD.[Baker *et al.* 1996;Hattori *et al.* 1998] It is of critical importance that both donor $CD4^+$ and $CD8^+$ T cells play a crucial role in the pathogenesis of acute GvHD, which is represented in cartoon form in Figure 1.2.

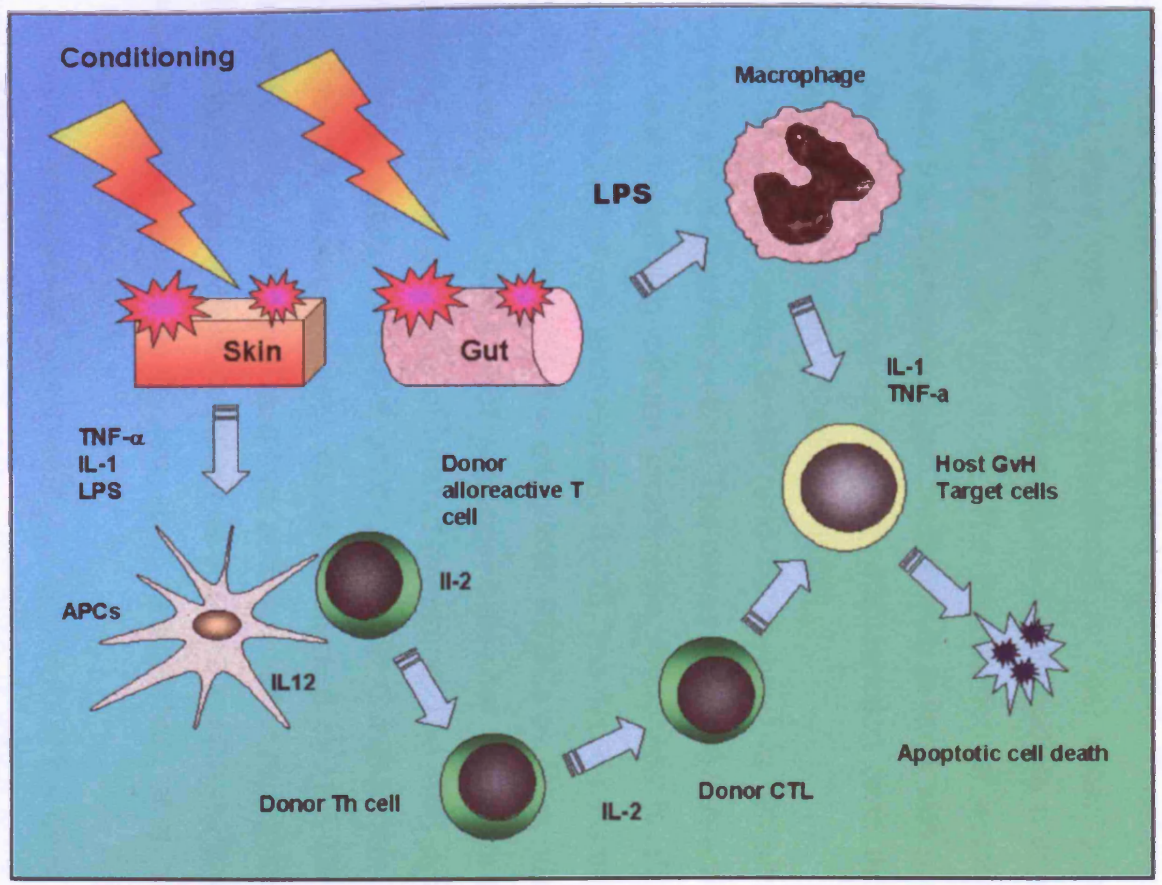

Figure 1.2 The three-step pathogenesis of GvHD.[Ferrara *et al* 1985]

Chronic GvHD is defined as GvHD occurring more that 100 days after AHSCT. It may supersede acute GvHD directly, develop after resolved acute GvHD or it may develop in the absence of any preceding GvHD. The clinical syndrome of chronic GvHD affects primarily the skin with the development of skin atrophy and changes resembling scleroderma. The liver and gut may be affected and the pulmonary syndrome of bronchiolitis obliterans is seen with a progressive restrictive lung defect. The multisystem changes seen in chronic GvHD have often been likened to those seen in autoimmune connective tissue disorders. There is some experimental evidence that alloreactive T cells in mice with chronic GvHD show specificity not restricted to host alloantigens but to a common MHC class II antigen epitope. Such T cells secrete a mixed cytokine profile (IL-4 and IFN-γ but not IL-2). The persistence of autoreactive IL-4 secreting T cells that promote collagen formation in patients with chronic GvHD has been linked to absent or impaired thymic function (and thus impaired deletion of autoreactive T cells). [Parkman 1986]

The risk of developing acute GvHD (and its severity) is difficult to predict, especially in the HLA-matched setting. The *mixed lymphocyte reaction* (MLR) is an in vitro test quantifying cellular proliferative responses to stimulation by allogeneic PBMCs.[DuPont and Hansen 1976] The MLR utilises γ-irradiated PBMCs as stimulatory cells and live allogeneic PBMCs as responder cells. The assay measures the proliferation of responder cells after 120 hours of co-culture by measuring their incorporation of tritiated thymidine. The MLR is useful in the HLA-mismatched setting to predict graft rejection or GvHD.[Odum *et al.* 1987] However the MLR does not detect responses evoked by HLA DP and mHag mismatches between donor and recipient, and has neither high positive nor high negative predictive value for the occurrence of GvHD in HLA-matched sibling or unrelated donor transplants.[al Daccak *et al.* 1990;Bishara *et al.* 1994;Bishara *et al.* 1999]

In an attempt to improve the predictive value for GvHD of the MLR in the HLA-matched sibling setting the MLR assay has been modified by both the pre-treatment of the stimulator cells with cytokines (TNF-α and IFN-γ) and the addition of stimulatory cytokines (IL-2 and/or IL-4) to the MLR co-culture itself. HLA-matched responder proliferation above and below 5% of that seen with mismatched stimulator cells was found to have fair positive and excellent

negative predictive value for acute GvHD after T replete full intensity conditioning sibling allogeneic transplantation (66% and 90% respectively).[Bishara *et al* 1994]

Using only IL-4 addition to the MLR co-culture, responder cell proliferation (RRI>5%) has been shown to be predictive of clinically significant GvHD in HLA A, B and DR-matched unrelated bone marrow transplants and to correlate with molecular HLA-C disparity between donor and recipient.[Bishara *et al* 1999]

Limiting dilution assays include the Cytotoxic T Lymphocyte Precursor (cTLP) and Helper T Lymphocyte Precursor (hTLP) assays. These assays quantify the frequency of $CD8^+$ and $CD4^+$ cells with activity in the GvHD direction. High cTLP frequency has been shown to correlate with HLA Class I mismatch and hTLP frequency with Class II mismatch although cTLP frequency cannot distinguish between Class I mismatches that cause GvHD and those that do not, and is thus not a reliable tool to predict clinical risk of GvHD.[El Kassar *et al.* 2001;Madrigal *et al.* 1997]

Another in vitro assay in use to predict GvHD is the *skin explant assay.* First described by Vogelsang in1985 this technique uses recipient skin as the target and donor PBMCs (which have been pre-stimulated with irradiated recipient PBMCs) as effector cells.[Vogelsang *et al.* 1985] Histopathological changes (akin to GvHD) in the skin biopsies are assessed after 72 hours of co-culture with donor PBMCs. The skin explant model has been shown to be a clear predictor of GvHD in adult HLA Class I and II matched non-T cell depleted sibling AHSCTs. In this setting the technique is better than limiting dilution assays (CTLp and/or HTLp frequency) in predicting GvHD, and has a positive predictive value as high as 91% in paediatric patients undergoing matched sibling transplants.[Dickinson *et al.* 1998;Hromadnikova *et al.* 2001;Sviland and Dickinson 1999]

More recently, polymorphisms genes encoding cytokines and cytokine receptors have been shown to be associated with increased risk of occurrence of both acute and chronic GvHD after sibling AHSCT. These include polymorphisms of the IFN-γ, IL-6, oestrogen receptor alpha and IL-1alpha genes. [Cavet *et al.* 2001;Cullup *et al.* 2003;Dickinson *et al.* 2001;Middleton *et al.* 2003] The relative importance of such polymorphisms in the HLA-mismatched or matched unrelated setting is yet to be determined.

Clinically severe GvHD occurs in 20-30% of non-T cell depleted HLA-matched sibling transplants and at least 40% of non-T cell depleted unrelated HLA-matched transplants[Chao and Schlegel 1995]. Treatment of established acute GvHD is ineffective and mortality is high.[Jacobsohn and Vogelsang 2004] Conventional treatment for established GvHD is highly immunosuppressive and delays immune reconstitution post AHSCT and the subsequent susceptibility to infection contributes significantly to the high mortality seen in recipients with severe GvHD. Various strategies have therefore evolved to prevent GvHD after AHSC transplantation.

Much work has focussed on the critical role of damage to the gut mucosa and LPS release. Gut decontamination with non-absorbable antibiotics has long been administered to recipients prior to AHSCT to reduce GvHD and there is evidence in both murine models and human trials of the efficacy of this strategy.[Lampert *et al.* 1988;Yabe *et al.* 1988]

The pharmacological blockade of LPS has also been shown to reduce GvHD in a mouse model.[Cooke *et al.* 2001] Murine work also supports the efficacy of IL-11 administration to polarise host T cells to Th2-type cytokine release and thus reduce GvHD.[Hill *et al.* 1998] A Phase I/II clinical study did not however support the use of IL-11 in adult AHSCT.[Antin *et al.* 2002] Keratinocyte growth factor has also been shown to reduce GvHD in murine experiments and is currently being used in a Phase I/II clinical trial in patients undergoing mismatched related or unrelated transplants.[Panoskaltsis-Mortari *et al.* 1998;Ratanatharathrn V *et al.* 2003] Both of these strategies have been shown not to adversely affect the graft-versus-leukaemia effect (*vide infra*) in murine models.

Reduction in intensity of recipient conditioning (especially the use of regimes that do not contain total body irradiation) theoretically should result in less GvHD by minimising host tissue damage, the initiating step in the pathogenesis of GvHD, and this has been reported in many published series of transplants utilising such *reduced intensity conditioning* regimes.[Bacigalupo 2002]

Anti TNF-□ antibody treatment administered during conditioning delayed the onset but did not prevent acute GvHD in a Phase I/II clinical study.[Holler *et al.* 1995] IL-2 also has a fundamental role in the pathogenesis of acute GvHD and when administered to recipients of autologous HSCT induces a GvHD-like syndrome.[Massumoto *et al.* 1996] However prophylactic anti-IL-2 antibody

treatment does not reduce the development of GvHD in the AHSCT and indeed when given as a treatment for acute GvHD worsened survival.[Blaise *et al.* 1991;Blaise *et al.* 1995]

The earliest pharmacological manoeuvres to prevent GvHD acted to suppress responsiveness of donor T cells either by *ex vivo* non-selective donor T cell depletion or by *in vivo* (post-transplant) donor T cell depletion/suppression by pharmacological treatments or antibodies administered to the transplant recipient.

The most commonly used pharmacological agents used to suppress donor T cells post transplant are drugs that share the common final pathway of action of inhibition of T cell activation. The longest established are the fungal derivatives *ciclosporin* and *tacrolimus*, which inhibit the calcineurin serine/threonine phosphatase that mediates transcription factor activation (e.g. NFAT) thus reducing the upregulation of pro-inflammatory cytokine production such as IL-2 by activated lymphocytes. Either of these two drugs is administered to the recipient post-transplant, often in conjunction with the dihydrofolate reductase inhibitor *methotrexate*. Initial use of these drugs developed historically after evidence of activity in animal studies and no randomised studies exist comparing the use of no GvHD prophylaxis with these pharmacological agents in human AHSCT. Several non-randomised trials have suggested benefit of pharmacological GvHD prophylaxis in non-T cell-depleted AHSCT. A landmark trial from Stanford, USA demonstrated that use of both ciclosporin and methotrexate (in conjunction with corticosteroids) halved the frequency of GvHD seen in matched sibling transplants given ciclosporin and steroids alone. A subsequent study in Spain demonstrated that corticosteroids as part of GvHD prophylaxis with ciclosporin and methotrexate were of no additional benefit and were possibly detrimental.[Ancin *et al.* 2001;Chao *et al.* 1993]

Animal studies have shown that tacrolimus treatment of $CD34^+$ haematopoietic stem cells promotes the development of dendritic cells that drive $CD4^+$ cells towards a Th2-type cytokine profile.[Shimizu *et al.* 2000] Indeed there is animal evidence that the myeloid growth factor granulocyte colony-stimulating factor (G-CSF) mobilizes Th2-inducing dendritic cells, which may help to protect against GvHD. It has been suggested that this effect underlies the relatively low incidence of acute GvHD seen allogeneic transplantation of G-CSF-mobilised

peripheral stem cell grafts, despite the presence of 10-20 fold more T cells than conventional bone marrow grafts.[Arpinati *et al.* 2000;Franzke *et al.* 2003]

Newer immunosuppressive agents include the TOR protein-binding P7056 kinase inhibitor *rapamycin*, which causes cell cycle arrest in G1, and the inosine monophosphate dehydrogenase inhibitor *mycophenolate mofetil* (MMF) that preferentially inhibits T cell activation as a result of selective purine metabolism in lymphocytes. Although the use of these two agents in both prophylaxis and treatment of GvHD is increasing, they are still mainly employed prophylactically when there is intolerance to ciclosporin and tacrolimus.

Anti-T cell antibodies have been employed as part of host conditioning regimes when there is a higher chance of graft rejection (aplastic anaemia, unrelated and mismatched transplants, reduced intensity conditioning regimes). Lympholytic concentrations of the humanised pan-lymphocyte anti-CD52 antibody *Campath 1H* can be detected for several days after transplantation when administration to the host as part of conditioning. There is no doubt that persistence of this antibody will cause a degree of depletion of donor T cells, and may serve to deplete recipient circulating (but not tissue) dendritic cells, which may abrogate GvHD by reducing indirect alloantigen presentation.[Buggins *et al.* 2002;Klangsinsirikul *et al.* 2002;Morris *et al.* 2003;Ratzinger *et al.* 2003]

1.5 Non-selective T Cell Depletion

Undoubtedly the single most effective method of preventing GvHD after AHSCT is donor T cell depletion prior to infusion of the graft into the recipient. This can be achieved either by the destruction of T cells within the graft or their removal by cell sorting techniques. Indeed the critical role of donor T cells in the pathogenesis of GvHD is underlined by the observation that the development of GvHD is related to the number of residual donor T cells left in the graft after T cell depletion, and that reduction of the infused T cell dose to $1x10^5$/kg recipient body weight effectively removes the incidence of severe GvHD in HLA-matched sibling AHSCT.[Atkinson *et al.* 1987] No additional pharmacological immunosuppression is required to prevent severe acute GvHD in this setting. T cell depletion has also been shown to be effective in GvHD prophylaxis in HLA-

matched or mismatched unrelated donor transplantation although some severe GvHD may still seen in this setting.[Ash *et al.* 1990]

The earliest methods used to deplete donor grafts of T cells included soybean lectin agglutination and sheep E rosette formation, which in combination leads to a 100-fold reduction in numbers of T cells within the graft.[Reisner *et al.* 1981]

The use of monoclonal antibodies in non-selective T cell depletion was pioneered in the early 1980s by Prentice et al. and Filipovich et al, with incubation of murine anti-CD3 (OKT3) antibody with (predominantly matched sibling) bone marrow grafts prior to reinfusion.[Filipovich *et al.* 1982;Prentice *et al.* 1982]

Other monoclonal anti-T cell antibodies have been used to T deplete grafts either alone or in combination and include anti-CD6 and the combination of anti-CD6, anti-CD7 and anti-CD8. [Prentice *et al.* 1984;Soiffer *et al.* 1997]

More recently dual selection procedures using immunomagnetic cell sorting systems (such as the CliniMACs system and the Isolex 300i device) using positive selection of $CD34^+$ cells followed by T cell depletion (by various methods) have been developed. These techniques lead to profound T cell depletion of grafts with a 3-5 log-reduction in T cell dose, often to below 10^4/kg recipient body weight, which is sufficient to prevent severe GvHD even in adult recipients of haplotype-mismatched related donor grafts.[Aversa *et al.* 2001]

Reduced rates of engraftment were seen in early reports of the use of T cell depleted HLA-matched sibling AHSC. Rates of engraftment comparable with non-T depleted HLA-matched sibling transplants were subsequently achieved by increasing either the dose rate (if given as a single fraction) or the total dose of irradiation (if administered in a fractionated regime) in the recipient conditioning regime, or the addition to recipient conditioning of immunosuppressive agents such as fludarabine and/or anti-T cell antibodies to reduce the number of host CTLps that mediate graft rejection. Depletion of the $CD8^+$ cell subset alone has been undertaken (in an attempt to improve post transplant immune reconstitution), and does reduce the incidence of GvHD in HLA-matched sibling donor transplants.[Nimer *et al.* 1994] However there is evidence that $CD4^+$ cells play a critical role in the pathogenesis of GvHD and when $CD4^+$ cells alone are given as Donor Lymphocyte Infusions (DLI), post

transplant at time of relapse, fatal GvHD has been reported suggesting that $CD4^+$ cells alone are capable of mediating severe GvHD.[Alyea *et al.* 1998]

An early series published by Horowitz et al demonstrated a significant increase in disease relapse in patients who received non-selective T cell depleted sibling allogeneic bone marrow transplants for leukaemia (when compared to those who received non-T cell depleted transplants).[Horowitz *et al.* 1990] This raised concerns about the concomitant loss of the anti-tumour effect that might also be mediated by donor T cells (the Graft-versus-Leukaemia effect, GvL) in non-selectively T cell depleted grafts. Horowitz also reported a lower relapse rate in recipients of non-T cell depleted grafts that developed GvHD suggesting that the allogeneic GvL and GvHD T cell responses were linked. However when only the transplant recipients with acute myeloid leukaemia are considered in this series, the increase in relapse in recipients who receive non-selectively T cell depleted grafts is greater than the number of recipients of non-T cell depleted grafts who developed GvHD, and so it could be postulated that allogeneic T cells exert a GvL effect in some recipients who do not develop GvHD. The latter observation suggests that the GvL effect and GvHD are to some degree separate processes, albeit with some common ground (underscored by shared alloantigens on recipient leukaemic cells and non-leukaemic tissues vulnerable to GvHD).

Further compelling evidence for the existence of the allogeneic GvL effect has been provided by the therapeutic efficacy of post-transplant DLI in treating relapse.[Kolb *et al.* 1995;Mackinnon *et al.* 1995] The GvL effect (as measured by overall response to DLI) is more marked in some diseases (e.g. Chronic Myeloid Leukaemia, (CML)) than others (e.g. Acute Lymphoblastic Leukaemia, Myeloma) and this probably reflects a combination of over-expression of tumour-restricted or tumour specific antigens in diseases susceptible to DLI, and the kinetics of disease progression.[Clark *et al.* 2001;Peggs *et al.* 2003a;Rezvani *et al.* 2003] Further evidence that the GvL effect and GvHD may be separable came from the ability of DLI, especially at low doses to induce disease remission without causing GvHD.[Mackinnon *et al* 1995]

Profound non-selective T cell depletion also leads to quantitative and qualitative delays in post-transplant immune reconstitution and to an increase in infections during this period.[Chakrabarti *et al.* 2002a;Chakraverty *et al.* 2001]

It is clear, therefore, that whilst non-selective T cell depletion is an effective way of abrogating GvHD in AHSCT, the loss of the GvL effect and increases in post-transplant infections are major disadvantages of this manoeuvre.

1.6 Selective Allodepletion

Some groups have reported the use of pharmacological agents or antibodies (such as butyric acid derivatives, T cell co-stimulatory blockade +/- anti-CD40 antibody, use of the proteasome modulator bortezomib) administered to the whole T cell population within the graft to reduce allogeneic responses. Although published reports describe successful abrogation of GvHD in MHC mismatched murine models there is little evidence presented for preservation of the GvL effect or cellular responses to infection and indeed no putative mechanism why these responses should be selectively retained using these techniques.[Blazar *et al.* 1997;Gilbert *et al.* 2003;Sun *et al.* 2004]

Genetic modification of T cells within the allogeneic haematopoietic stem cell graft has also been undertaken to permit pharmacologically triggered inhibition (or destruction) of donor $CD8^+$ cells, to be employed when GvHD develops. The reinfusion of donor $CD8^+$ cells transduced with the herpes simplex thymidine kinase enzyme (which allows them to be selectively destroyed by administration of *ganciclovir* to the recipient) is the most elegant of these systems but has proved a difficult one to develop to clinical grade and is also limited by cell cycle dependence and increased immunogenicity of transfected T cells.[Helene *et al.* 1997;Riddell *et al.* 1996]

The triggering of apoptosis via the intracellular FAS/FKBP-mediated pathways in $CD8^+$ cells causing GvHD is an alternative approach which has been shown to eliminate >80% of human transduced $CD8^+$ cells regardless of their proliferation status.[Thomis *et al.* 2001] However, these methods lack specificity, and when employed are not restricted to alloreactive T cell inhibition and/or destruction.

An alternative approach to reducing GvHD, whilst potentially retaining cellular responses to infection (and possibly tumour cells) is the identification and subsequent removal/disablement of T cells within the graft able to recognise and respond to host alloantigen stimulation. This process is called selective T cell depletion or *allodepletion.*

All published techniques of selective allodepletion of haematopoietic cells utilise a common fundamental mechanism. Stimulator (usually irradiated) cells capable of presenting alloantigens are incubated with live HLA-mismatched (or matched) responder cells in a mixed lymphocyte reaction. T cells within the responder cell population that are specific to stimulator alloantigens are subsequently identified and either rendered anergic, destroyed or removed, depending upon the individual technique. The resulting *allodepleted* responder cell fraction is available for re-infusion to the transplant recipient.

Many individual techniques have been described for achieving selective allodepletion of allogeneic haematopoietic stem cell grafts and can broadly be divided into three categories:

1. Induction of anergy in alloreactive T cells (e.g. by CTLA-4 antibody administration to the MLR).

2. Identification of alloreactive T cells by their increased metabolic activity or proliferative rate and their subsequent destruction or depletion using the techniques of photodynamic purging or carboxyfluorescein diacetate succinimidyl ester (CFSE) dye dilution.

3. Identification of alloreactive T cells by their cell surface expression of antigens associated with T cell activation and their subsequent destruction or depletion. (e.g. CD25-linked immunotoxin techniques, CD69 immunomagnetic depletion, CD95 ligation).

Different mechanisms utilise different MLR conditions and different APCells and some individual techniques have been investigated using several APCells. The optimal choice of APCell and individual co-culture conditions in the MLR may be different for each technique and may also differ when allodepletion techniques are applied clinically, depending on the degree of HLA mismatch between donor and recipient and the disease groups of recipients. A cartoon illustrating the various techniques that have been published for selective alloreactive T cell depletion is shown in Figure 1.3. It also shows the range of APCells that may be used to present recipient alloantigens to donor (responder) cells in the MLR.

The published evidence for the use of each individual technique is described in detail in subsequent sections.

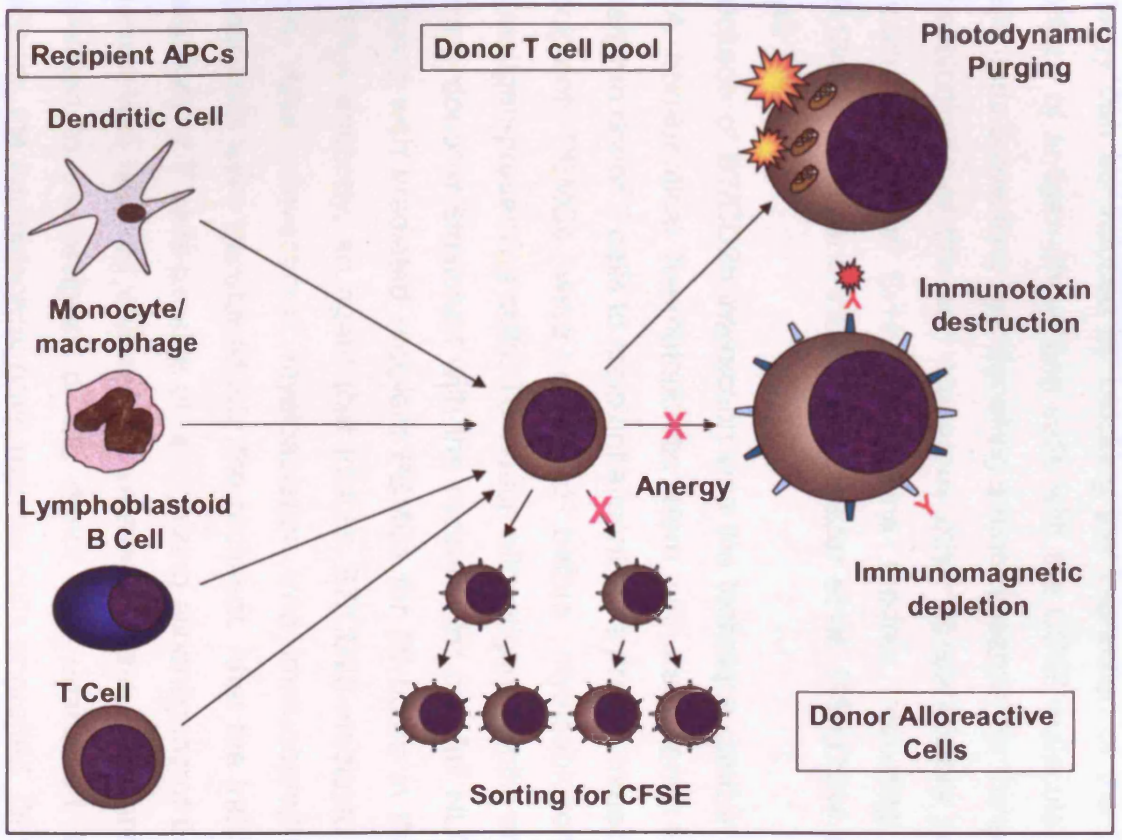

Figure 1.3 Techniques available for selective allodepletion of haematopoietic stem cell grafts and the range of antigen presenting cells used in such techniques.

1.7 The Induction of Anergy In Alloreactive T Cells

An alternative to depletion or destruction of alloreactive cells is the induction of anergy in such cells. The presentation of antigens in a one way (host to graft) mixed lymphocyte reaction leads to the activation of alloreactive cells within the graft unless co-stimulatory blockade is employed: in this situation the result is the induction of hyporesponsiveness or anergy on host reactive donor cells. Such anergy can be induced by blocking the interaction of the B7 molecule on the surface of antigen-presenting cells with the CD28 molecule on the surface of T cells, thus preventing key signalling events essential for the activation of T cells. The blockade of this and numerous other co-stimulatory pathways have been shown to reduce GvHD in murine models, including CD40/CD154 and CD134/CD134 ligand interactions.[Blazar *et al.* 1994;Durie *et al.* 1994;Tsukada *et al.* 2000]

Blockade of B7/CD28 interaction was the technique used in a clinical trial in 12 HLA non-identical haematopoietic stem cell transplants for the induction of anergy in donor T cells to recipient alloantigens prior to infusion into the recipient. Recipient PBMCs were collected before myeloablation and served as alloantigen-presenting cells. To induce alloantigen-specific anergy, bone marrow from a donor mismatched with the recipient for one full HLA haplotype was co cultured with irradiated recipient PBMCs for 36 hours in the presence of anti-CTLA-4 antibody, an agent that inhibits B7/CD28-mediated co-stimulation of T cells. After conventional myeloablation and immunoprophylaxis, the treated donor cells were transfused into the recipient. After the induction of anergy, the frequency of T cells capable of recognizing alloantigens of the recipient in donor marrow was reduced whereas the responsiveness to alloantigens from persons unrelated to the recipient or the donor was unaffected. In all 11 evaluable patients, the haploidentical bone marrow cells engrafted. Of these 11 patients, 3 had acute GVHD confined to the gastrointestinal tract. No deaths were attributable to GVHD. Five of the 12 patients were alive and in remission 5 to 29 months after transplantation. This trial seems to support the technique of induction of anergy in alloreactive cells by co-stimulatory blockade, although it must be noted that several of the recipients were in frank haematological relapse pre-transplant and thus the majority of the cells used to present alloantigens in the donor co-culture were leukaemia cells. If leukaemia-restricted (or-specific)

antigens were presented to donor T cells and subsequently rendered anergic then the anti-leukaemic activity (GvL) of the AHSC graft would be reduced with the possibility of increased disease relapse. It is therefore of note that no long-term follow-up of patients on this trial has been published.[Guinan *et al.* 1999]

1.8 Antigen-independent Selective Allodepletion Techniques

1.8.1 Photodynamic Purging

Photodynamic purging (PDP) of alloreactive cells is a technique that utilises the increased metabolic activity seen in alloreactive donor T cells when stimulated by recipient alloantigens, and has been demonstrated in a non-identical murine model. Donor cells were stimulated with irradiated BALB/c (H-2 (d)) host spleen cells in a 5-day mixed lymphocyte culture. Following this activation, a photoactive rhodamine derivative called 4,5-dibromorhodamine 123 (TH9402), was added. This compound is selectively retained in the mitochondria of activated host-reactive cells (but not tumour- or third-party-specific resting cells). Treated cells were subsequently exposed to visible light (514 nm) to deplete the TH9402-enriched activated alloreactive cells. Selectively allodepleted T cells were subsequently infused into C57BL/6 (H-2 (b)) mice. Treatment with photodynamic cell purging process inhibited antihost responses (measured by cTLP by 93%, and IFN-γ production by 66%). By contrast, anti-BCL1 (BALB/c-origin leukaemia/lymphoma) and anti-third-party C3H/HeJ (H-2 (k)) responses were preserved. PDP-treated primed C57BL/6 cells were also further tested in vivo. All lethally irradiated BALB/c mice inoculated with BCL1 leukaemia/lymphoma cells and T cell-depleted bone marrow cells developed leukaemia with a 50% mortality by 100 days. All mice died of GvHD after addition of 5 x 10^6 untreated primed C57BL/6 bone marrow cells. However, addition of same numbers of PDP-treated C57BL/6 bone marrow cells allowed 90% of the recipients to survive more than 100 days without detectable BCL1 tumour cells and free of GvHD. Moreover, PDP-treated primed C57BL/6 cells retained the ability to induce GvHD in third-party (C3H/HeJ) mice.[Chen *et al.* 2002]

1.8.2 CFSE Dye Dilution Techniques

Donor cells may be labelled prior to allostimulation in the MLR with 5-(and 6-) CFSE, which is diluted out when cells divide. The $CFSE^{bright}$ (non-dividing) cells after allostimulation in the MLR may be sorted flow cytometrically, a positive selection system of non-alloreactive cells. This technique has been used in an in vitro human model using HLA-mismatched PBMC stimulators. After flow cytometric sorting of $CD4^+$ $CFSE^{bright}$ responders, proliferative responses to original allostimulator PBMCs were reduced, much more markedly so (90-95% reduction) if responder cells were depleted of cells expressing the activation marker CD25 (*vide infra*).[Godfrey *et al.* 2004] The reduction in proliferative responses after alloreactive cell depletion using this technique was improved when purified matured monocyte-derived dendritic cells (DCs) were used as stimulators (a 100-fold (99%) reduction in alloreactivity was attained). Significantly, the $CFSE^{bright}$ $CD25^-$ cells recovered from these cultures retained responses to Candida and cytomegalovirus (CMV) antigens. In addition, the CFSE-based approach prevented GvHD in a MHC Class II disparate murine model. An obvious criticism of this technique is that when allodepletion is based on CFSE alone, only proliferating $CD4^+$ cells are removed and any alloreactive response in non-proliferative cytokine secreting $CD4^+$ and $CD8^+$ cells will remain. This presumably explains the further reduction in the alloresponse when CFSE technique was combined with selection of cells negative for the activation marker CD25. Cell sorting by flow cytometry is a difficult technique to perform under sterile conditions and thus would be potentially difficult to develop to a clinical scale.

1.9 Antigen-dependent Selective Allodepletion

1.9.1 CD95

Huber's group recently described a technique of selective allodepletion that utilizes the upregulation of the FAS receptor (CD95) upon alloreactive T cells and activation-induced cell death (AICD) (by apoptosis) caused by antibody-mediated ligation of the FAS receptor on such cells in a MHC mismatched murine model. Activation of resting or pre-activated T lymphocytes from C3H/HeJ (H-2 (k)) mice was induced in an MLR with irradiated BALB/cJ (H-2 (d)) mouse-derived stimulators. A substantial decrease (80%) of proliferative and lytic responses by activated alloreactive T cells was subsequently achieved by incubating the MLR with an agonistic monoclonal antibody to CD95, and residual T cells recovered did not elicit alloreactivity upon challenge to H-2 (d). Depletion of alloreactive T cells by AICD was shown to be specific to host alloantigens because reactivity to an I-A (d)-restricted ovalbumin (OVA) peptide by OVA-specific $CD4^+$ T cells mixed into the allogeneic T cell pool and subjected to induction of AICD in the absence of OVA peptide could be preserved. Adoptive transfer of donor-derived allogeneic T lymphocytes, depleted from alloreactive T cells by AICD in vitro, in the parent (C3H/He) to F (1) (C3H/He x BALB/c) GvHD model prevented lethal GvHD.[Hartwig *et al.* 2002]

1.9.2 CD25

CD25 is a 55KD glycoprotein and forms the □-chain low affinity component of the IL-2 receptor. Surface expression of CD25 is increased on T and B cells upon activation when the high affinity trimolecular IL-2 receptor complex is formed.[Smith 1984]

The earliest report of selective alloreactive cell depletion in a human in vitro model utilised the upregulation of the CD25 antigen on alloreactive cells, published in 1990. After a two-day MLR with HLA-mismatched human PBMCs as stimulators, activated host alloreactive (blood or bone marrow) T cells were incubated with a ricin A-chain toxin conjugated with the antibody 33B3.1 directed against the CD25 receptor. Complete inhibition of proliferation in the MLR to original HLA-mismatched stimulators was seen, and reduction of cytotoxic

responses was also observed. This method only modestly reduced the alloreactivity of blood or marrow T cells toward third party HLA-mismatched stimulators. Limiting-dilution analysis of residual alloantigen-reactive T lymphocytes showed that this technique resulted in a twenty fold to fifty-fold reduction of antihost reactivity.[Cavazzana-Calvo *et al.* 1990]

This technique was used by the group in an H-2 haplotype disparate murine transplantation model and was found to reduce pathological changes of gut GvHD. It was also shown to increase survival in such mice, one third being alive at 100 days (by which time all the mice give unselected grafts had died). [Cavazzana-Calvo *et al.* 1994] Preservation of anti-leukaemia and antiviral activity after selective allodepletion using this technique was subsequently evaluated by limiting dilution assays measuring the frequency of CTLp directed against autologous leukaemia blasts (LB) and CMV- and Epstein-Barr virus (EBV)-infected target cells. Anti-leukaemia activity was evaluated in PBMC of 3 patients treated for acute myeloid leukaemia, who had developed a high frequency of LB-reactive CTLp after either autologous or allogeneic BMT. Depletion with CD25 immunotoxin efficiently inhibited proliferation to original HLA-mismatched stimulators in the MLR and effector cells obtained after allodepletion fully retained the capacity to lyse LBs. In contrast, the frequency of CTLp directed against patient's pre-transplant bone marrow remission cells was always undetectable. Data from 4 healthy donors showed that specifically allodepleted T cells recognized and killed autologous CMV-infected fibroblasts and autologous EBV-B-lymphoblastoid cell (LBC) lines [Montagna *et al.* 1999]

This technique has subsequently been developed to a clinical scale in a phase 1/2 study, in which 1-8 x 10^5 allodepleted T cells/kg were infused between days 15 and 47 into 15 paediatric patients who had acquired or congenital haemopoietic disorders and who received CD34-selected AHSCT on day 0 (with no additional GvHD prophylaxis). Most transplants were HLA non-identical family members and some were full haplotypical mismatches. Less than 1% residual anti-host alloreactivity was recorded in 12 of 16 procedures. No cases of severe GvHD arose, although it must be note that the doses of allodepleted T cells the patients received were relatively small, (although a dose of unselected T cells of >10^4/kg is sufficient to induce GvHD in a haplotypic transplant). The cases where GvHD was seen were recipients of grafts that retained some anti-host reactivity in the MLR after selective allodepletion.[Andre-Schmutz *et al.* 2002]

The efficacy of this technique in vitro has been confirmed by other groups.[Datta *et al.* 1994;Harris *et al.* 1999] The Pseudomonas exotoxin-based immunotoxins, anti-Tac (Fv)-PE38 and anti-Tac (Fv)-PE38KDEL, (both targeting the IL-2 receptor on activated T cells), has been used to specifically deplete alloreactive lymphocytes against haploidentical stimulators. Mean residual reactivity (proliferative responses) after depletion was 8% of that seen pre-depletion against the haploidentical stimulator and 65% against third party stimulators. The immunotoxin did not affect growth of normal BM cells.[Mavroudis *et al.* 1996] This technique has been extended to fully HLA-matched pairs (in patients with chronic myeloid leukaemia and their HLA-matched sibling donors). Donor hTLP frequencies were measured against recipient PBMCs, recipient LBs and third-party PBMCs, before and after depletion. There was a 4.3-fold reduction of donor-versus-host hTLP frequency without a significant change in the donor frequencies against third party PBMCs. There was a smaller non-significant 1.8-fold reduction in donor-versus-leukaemia hTLP frequency.[Mavroudis *et al.* 1998] This technique has been developed to a clinical scale, in which stimulator cells were generated from immunomagnetically selected and OKT3-expanded recipient T lymphocytes. Stimulator cell selection/expansion yielded high numbers of enriched $CD3^+$ cells. After selective allodepletion, cell recovery and viability was high, permitting a selectively allodepleted T cell dose > 1×10^8 $CD3^+$ cells/kg to transplant recipients. Depletion efficiency and reduction in proliferative responses to original stimulators were akin to the smaller scale in vitro work this group have published. Furthermore cellular responses to staphylococcus enterotoxin B and CMV Ag (measured by IFN γ secretion) were largely retained.[Solomon *et al.* 2002] A phase I study utilising this technique of selective allodepletion is underway in HLA-matched sibling adult donor-recipient pairs, with reduced intensity recipient conditioning. Preliminary results have now been reported in abstract form.[Solomon *et al.* 2003] Eight patients with advanced haematological malignancies with a median age of 66 years have received selectively allodepleted grafts from sibling donors after reduced intensity conditioning. The median $CD3^+$ cell dose was 1.1×10^8/kg recipient body weight. Ciclosporin was used as additional GvHD prophylaxis. Acute GvHD requiring treatment occurred in 6 of the 8 patients, although severe acute GvHD was seen in only one recipient. T cell recovery post-transplant was equivalent to T replete

historical controls, and 5 were free of disease with a median follow up of 5 months.

This technique has recently been modified using an alternate system of recipient antigen presentation, using recipient EBV-transformed LCLs as stimulators to activate donor alloreactive T cells. Allodepletion with an anti-CD25 immunotoxin following stimulation with HLA-mismatched host LCLs more consistently depleted in vitro alloreactivity than stimulation with host PBMCs, (as assessed by proliferation in MLR pre- and post-depletion) Allodepletion using this approach specifically abrogated cytotoxic T cell responses against host LCLs in IFN-γ enzyme-linked immunospot (ELISPOT) assays, whereas antiviral responses to adenovirus and CMV were largely preserved in similar assays following allodepletion. Donor anti-EBV responses were partially retained by recognition of EBV antigens through the non-shared haplotype. The frequency of T cells recognizing the PR1 epitope of proteinase 3 (an antigen over-expressed on CML cells) assayed by specific tetramer binding, was not significantly reduced in allodepleted cells from patients with CML.[Amrolia *et al.* 2003a]

A phase 1 clinical trial of infusion of selectively allodepleted donor T cells after haploidentical stem cell transplantation is underway in paediatric patients and preliminary results have been presented only in abstract form.[Amrolia *et al.* 2003b] 9 patients with relapsed/refractory haematological malignancies have received allodepleted T cells after disease-specific conditioning (reduced intensity in 2 recipients) from haploidentical donors. 6 recipients received 10^4 and 3 patients 10^5 allodepleted T cells/kg body weight at day 50, 80 and 110 post transplant. At a median follow up of 9 months 8 recipients were alive and 5 were disease-free. 2 recipients (one at each dose level) developed severe GvHD. Immune reconstitution was improved (compared to historical controls) in the recipients of the higher dose of allodepleted T cells.[Amrolia *et al* 2003b]

It is important to note that selective allodepletion based on CD25 expression will remove all cell expressing CD25 (not just those expressing CD25 upon allostimulation). Cells that constitutively express CD25 include $CD4^+CD25^+$ T regulatory cells, now recognized as playing an important role in the pathogenesis of GvHD. $CD4^+CD25^+$ T regulatory cells are required for the ex vivo induction of tolerance to alloantigen via co-stimulatory blockade and to inhibit allogeneic skin graft rejection. Depletion of $CD4^+CD25^+$ cells from the donor T cell inoculum resulted in increased GVHD mediated by $CD4^+$ or whole T cells fractions in mice.

The infusion of high numbers of purified $CD4^+CD25^+$cells donor cells significantly inhibited rapidly lethal GvHD.[Taylor *et al.* 2002] Thus there are concerns that removal of alloreactive cells based on induced expression of CD25 will also remove $CD4^+CD25^+$ T regulatory cells and that the absence of the T regulatory cell component in such manipulated AHSC grafts may disrupt immune reconstitution, (and could actually favour the development of GvHD).

1.9.3 CD69

CD69, (previously known as activation inducer molecule, very early activation antigen, MLR-3 and Leu-23) is a Type II transmembrane glycoprotein and a member of the natural killer (NK) cell gene complex family of signal transducing receptors.[Ziegler *et al.* 1993] CD69 has a C-type lectin-binding domain in the extracellular portion of the molecule, the crystal structure of which has been described. CD69 is constitutively expressed on human monocytes, platelets and epidermal Langerhans cells, whilst expression of CD69 is induced in vitro on activated cells of most haematopoietic lineages, including T and B lymphocytes, NK cells, murine macrophages, neutrophils and eosinophils, Although the gene for CD69 has been identified and cloned, the specific ligand for CD69 has not been identified.[Santis *et al.* 1994] The wide cellular distribution of CD69 and the induction of intracellular signals upon CD69 cross-linking suggest a role for the receptor in the biology of haematopoietic cells.[Marzio *et al.* 1999]

The function of CD69 on T lymphocytes is well established, acting as a signal transmitting receptor and co-stimulatory molecule resulting in proliferation and cytokine secretion. More recently data supporting a role in T cell development, particularly thymic emigration have been published, although CD69 knock-out mouse models did not show any functional impairment of T cells.[Borrego *et al.* 1999;Feng *et al.* 2002;Lauzurica *et al.* 2000;Nakayama *et al.* 2002]

Data are also emerging regarding the role of CD69 expression on NK cells. It has been shown that CD69 triggers NK-cell-mediated cytolytic activity, and the interaction of CD69 ligation with other C-lectin type NK inhibitory receptors (e.g. CD94) is under investigation.[Borrego *et al.* 1999]

CD69 also functions as a transmembrane signal transduction molecule in both neutrophils and platelets.[Testi *et al.* 1994] CD69 expression is inducible on T cells and the magnitude and kinetics of the CD69 response to polyclonal

mitogens and other antigenic stimuli are well described. CD69 expression on T cells is rapid, typically occurring within 4hrs in response to Staphylococcal Enterotoxin B, Tetanus toxoid and OKT3, peaking after stimulation with the latter at 48 hours.[Simms and Ellis 1996]

CD69 is expressed earlier on normal T cells stimulated with mitogens or antigens than other inducible activation antigens (such as CD25, CD71 and HLA DR).[Caruso *et al.* 1997] Although most (but not all) data published supports a relationship between the magnitude of the CD69 response on T cells and subsequent T cell proliferation (in tritiated thymidine assays), this relationship has not been found to be a direct linear correlation, and may vary between specific antigenic stimuli. Some reports have noted that the CD69 response to low dose antigenic stimulus is more sensitive than proliferative responses.[Caruso *et al.* 1997;Prince and Lape-Nixon 1997;Simms and Ellis 1996]

CD69 expression on T cells induced by HLA-mismatched allogeneic stimuli has been described. In the first of such reports, HLA-mismatched allogeneic PBMC induced CD69 expression on 6-13% of $CD3^+$ cells between days 3-6.[Simms and Ellis 1996] Leiva described equivalent T cell CD69 responses to allogeneic PBMC sonicates and irradiated allogeneic PBMCs, (with mean CD69 expression in 7% of $CD3^+$ HLA-mismatched responders, and a significant correlation between CD69 expression and proliferation (thymidine uptake at day 6), but 'with significant individual variations'.[Leiva *et al.* 1997]

CD69 expression on responding T cells after HLA-mismatched allostimulation by PBMCs occurs later than CD69 expression in response to mitogens, (peaking at 72 hours but showing a second increase in expression at 120 hours). Expression of CD69 in response to HLA-mismatched PBMC allostimulation occurs earlier than CD25 expression.[Craston *et al.* 1997]

Although some data have been published describing phenotypic characteristics of T cells expressing CD69 in response to mitogen, no reports describe such data in cells expressing CD69 in response to allogeneic stimuli.[Rutella *et al.* 1999]

Alloreactive cells have been identified by their expression of CD69 after allogeneic stimuli and specifically depleted by immunomagnetic sorting. Koh et al described an in vitro model in which HLA-matched donor PBMCs were co-cultured with irradiated recipient cells, which had been pre-stimulated with cytokines (IFN-γ and TNF-α) in a modified MLR. In 5 pairs, mean proliferative

responses post-depletion were 11.5% of the pre-depletion response to the original matched stimulators, (while the post depletion third-party responses were largely preserved at 80% of the pre-depletion value.[Koh *et al.* 1999]

The CD69 allodepletion strategy has been tested in a non-obese diabetic/severe combined immunodeficient (NOD/SCID) murine model of aggressive GvHD. Recipients of infusions of non-manipulated MHC class I and class II-mismatched donor T cells suffered rapid onset of acute, and generally fatal, GvHD. (The model was akin to aggressive clinical transfusion-related GvHD). Donor splenocytes were stimulated with irradiated recipient mononuclear cells in an MLR and subsequently depleted of $CD69^+$ alloreactive donor cells ex vivo by immunomagnetic sorting. The depleted donor cells were infused into sub-lethally irradiated recipient mice, monitored for 10 weeks post infusion. Mice receiving selectively allodepleted donor cells demonstrated significantly improved survival (71.4% compared with 12.5% in recipients of unmanipulated donor T cells, Figure 1.4) and absence of clinical GvHD, despite the presence of circulating donor lymphocytes demonstrated by flow cytometry.[Koh *et al.* 2002] The efficacy of selective allodepletion based on CD69 expression has been confirmed in vitro models by other groups. Fehse et al used a fibroblast enriched stromal layer of recipient cells as an antigen presentation system for allogeneic stimulation of donor cells. Alloreactivity of donor T cells (assayed as MLR proliferative responses and hTLP frequencies) was significantly decreased after depletion using magnetic cell sorting of cells expressing either CD25 or CD69. Interestingly the lowest level of post depletion alloreactivity was found when both $CD25^+$ and $CD69^+$ cells were depleted. No data have been published describing whether CD69 and CD25 are co-expressed on alloreactive T cells or whether these antigens are expressed on different subsets of alloreactive cells.[Fehse *et al.* 2000a;Fehse *et al.* 2000b]

Selective depletion of donor alloreactive cells by immunomagnetic sorting of $CD69^+$ cells stimulated by recipient keratinocyte cells has also been reported in an attempt to differentially remove cells with alloreactivity (resulting from tissue-specific mHag disparity) directed against skin.[van Dijk *et al.* 1999] No data have been published confirming the preservation of anti-leukaemic responses after allodepletion based on CD69 expression.

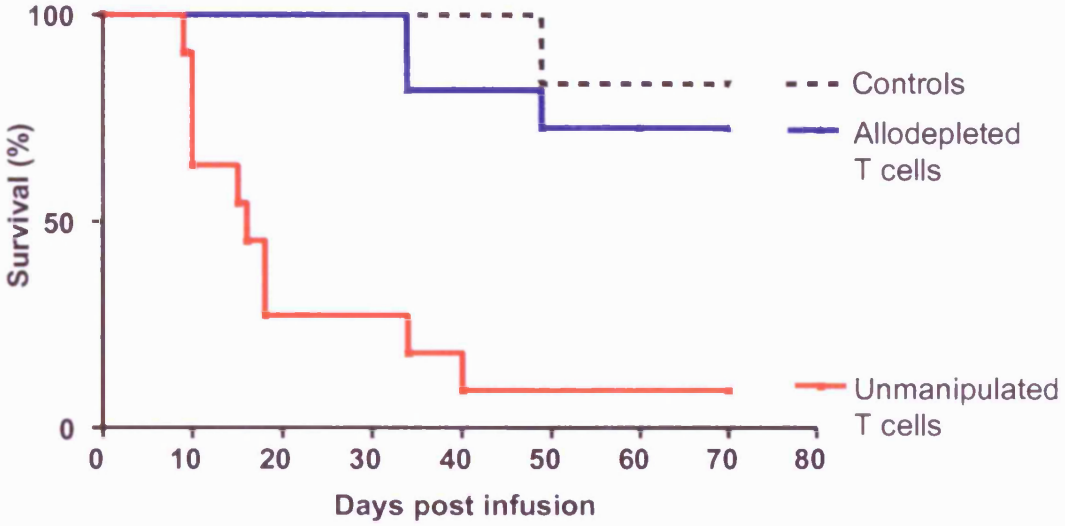

Figure 1.4 Selective depletion of $CD69^{+}$ cells and effect on survival of NOD/SCID recipient mice. Recipient mice received either phosphate buffered saline (controls, n = 18), 5-10x10^{6} non-manipulated fully MHC-mismatched T cells (n = 27) or 5-10x10^{6} allodepleted fully MHC-mismatched T cells (n = 14). Survival curves were compared using the log-rank test. Comparison of the survival curve of the control cohort with that of the mice receiving allodepleted T cells was not statistically significant. In contrast the survival of controls and of recipients of allodepleted T cells were both significantly better than that of recipients of non-manipulated T cells (P<0·0001 and P< 0·001 respectively). Figure adapted from Koh et al.[Koh, Prentice, Corbo, Morgan, Cotter, and Lowdell 2002]

1.10 Clinical Application

The ultimate test for the safety and efficacy of the CD69 allodepletion technique (in terms of abrogating severe GvHD and improving immune reconstitution) is the development of the technique to a clinical scale and its subsequent application in a Phase I clinical trial in adult AHSCT.

1.11 Aims of this Thesis

1. To evaluate an alternative antigen presentation system (the use of OKT3 pre-treated PBMCs) as stimulator cells in the MLR prior to CD69-mediated allostimulation.
2. To study residual antiviral responses in CD69 selectively allodepleted cells using a variety of alloantigen presentation systems.
3. To study in depth the phenotype of alloreactive and non-alloreactive cells based on CD69 expression.
4. To study the co-expression (and kinetics of expression) of CD69 and CD25 on alloreactive T cells and the effect of CD69-mediated allodepletion on the number and function of $CD4^+CD25^+$ T regulatory cells.
5. To investigate the possibility of T cell cross reactivity for alloantigens and CMV antigens.
6. To develop the CD69 mediated allodepletion technique to a clinical scale and instigate a Phase I clinical study of the use of CD69-selectively allodepleted T cell re-infusion in HLA-matched sibling haematopoietic stem cell transplantation for adult patients with acute myeloid leukaemia.

Chapter 2 General Materials and Methods

General materials and methods are described herein. Specific materials and methods only relevant to individual results chapters are described at the beginning of the relevant chapter.

2.1 Cell Culture

2.1.1 Reagents for Cell Culture

All cell culture was undertaken in "complete medium", which consisted of Roswell Park Memorial Institute hydrogen-carbonate-buffered medium (RPMI1640, Gibco/Life Technologies Ltd,) with 10% Foetal Calf Serum (FCS, Gibco) or 10% human AB serum (North London Blood Transfusion Centre) where stated, and 50units/ml Penicillin + 50μg/ml Streptomycin (Gibco/Life Technologies Ltd). Human cells for incubation and hybridoma cell lines were suspended in complete medium at 0.5-1x10^6 cells/ml in 24 or 96 well plates, 80 ml or 200ml tissue culture flasks (Nunc), and Lifecell 160cm^3 or OptiCyte 390cm^2 gas permeable bags (Baxter). All incubations were at 37°C, 5% CO_2 and 60% humidity unless otherwise stated.

2.1.2 Centrifugation

All centrifugation of tubes was undertaken on a Beckman GP machine (Beckman-Coulter) with a rotor arm length of 22.5cm, upon which a spin rate of 1500rpm equates to 400g and 1000rpm to 200g. Centrifugation of cell culture bags was performed at 200g in a Rotina temperature controlled centrifuge (Hettich), which was programmable directly for g force.

2.1.3 Isolation of Fresh Human Peripheral Blood Mononuclear Cells

Fresh peripheral blood samples were collected into 50ml plastic tubes (Nunc) with 100μl preservative free heparin/10 mls blood. Samples were diluted 1:1 in Hanks' Balanced Salt Solution (HBSS, Gibco/Life Technologies). PBMCs were isolated by discontinuous density gradient centrifugation. Diluted blood was

layered onto an equal volume of Ficoll (Nycomed Pharma AS) into 50 ml Falcon tubes (Nunc) at room temperature and spun at 400g for 25 minutes with no brake. The isolated mononuclear cell fraction was recovered at the interface with a sterile Pasteur pipette, diluted in complete medium and centrifuged for a further 10 minutes at 400g. The supernatant was discarded and the cell pellet was then gently disrupted manually with a sterile Pasteur pipette and resuspended in 1-4mls of fresh complete medium depending on the size of the cell pellet.

2.1.4 Cell Counting and Viability Assessment

10µl of PBMC suspension was pipetted into a 0.5ml microcentrifuge tube (Nunc). 90µl of 0.4% Trypan Blue (Sigma) stain (which is extruded by viable cells but taken up by non-viable cells) was added, making a 1:10 dilution (the final concentration of Trypan Blue was 0.36%) and thoroughly mixed. A bright-line haemocytometer (Sigma-Aldrich) was loaded with 10µl cell mixture (Trypan Blue and PBMCs) under a glass cover slip. The cell suspension was allowed to settle for 30 seconds in the haemocytometer and cells were counted with any part within the central 25 large squares (excluding cells that touched the bottom or right vertical perimeter line) using a phase contrast microscope (Nikon). The number of PBMCs/ml was then calculated as:

$$\text{PBMC } (10^6/\text{mL}) = \frac{\text{PBMCs in all 25 squares} \times 10^4}{25}$$

To calculate cell viability:

$$\%\ \text{Viability} = \frac{\text{Number of clear (viable) cells counted} \times 100}{\text{Number of Clear (viable) and blue (non-viable) cells counted}}$$

Unless otherwise specified, cells were resuspended and used at concentrations of 10^6 viable cells/ml complete medium.

2.1.5 Cryopreservation of Cells

For cryopreservation of cells 'cryomed' freezing medium was made up with 80% FCS (or human AB plasma) and 20% dimethylsulphoxide (DMSO, Sigma-Aldrich) and stored in aliquots at -20°C for up to one year. Cells for cryopreservation were brought to a minimum concentration of $10x10^6$/ml and mixed drop by drop with an equal volume of cryomed freezing medium on ice. The mixture was transferred immediately to cryopreservation vials (Nunc) in 0.5ml aliquots and the vials placed in isopropyl alcohol freezing containers (Nalgene) for controlled rate freezing and placed in a –70°C freezer overnight. The vials were then transferred to storage in liquid nitrogen at –196°C.

For resuscitation of cryopreserved cells, cryopreservation vials were transferred from liquid nitrogen on ice to a 37°C water bath. Cryopreservation vials were partially immersed and gently agitated until only a small frozen part remained. Cryopreservation vials were then wiped with alcohol and the contents pipetted immediately into a 30-fold volume of complete medium, pre-warmed to 37°C and then centrifuged at 200g for 10 minutes. The resulting cell pellet was gently disrupted manually with a pipette and resuspended in fresh complete medium for counting and viability assessment.

2.2 Flow Cytometry

2.2.1 Antibody Staining Protocols for Flow Cytometric Analysis

Unless otherwise stated antibodies used were supplied from Becton Dickinson (BD) and used at a volume of 10µl/10^5 viable cells. Polystyrene falcon tubes (BD) were used for staining of cells. For staining of PBMCs 100µl of PBMC cell suspension at 10^6/ml was incubated with 10µl of each of up to 4 fluorochrome-conjugated primary antibodies for 15 minutes at room temperature (unless otherwise stated) in the dark. (Simple titration experiments confirmed adequate cell labelling with 10µL of BD antibodies per 10^5 cells except BD PerCP fluorochrome conjugated antibodies, where 5µL of antibody adequately labelled 10^5 cells). Cells were then diluted in 500µl HBSS and centrifuged at 400g for 10 minutes and the cell pellet resuspended in 300µl of HBSS. Samples were kept

at 4°C and acquired within 12 hours of antibody staining. Antibodies used were conjugated to the following fluorochromes; Fluorescein isothiocyanate (FITC), Phycoerythrin (PE), Peridinin Chlorophyll Protein (PerCP) and Allophycocyanin (APC). If an unconjugated murine primary antibody was used to label cells, the initial staining procedure was identical to that described above. After staining with primary antibody a repeat washing step was added. 10μL of secondary fluorochrome conjugated goat (or rat) anti-mouse Fc-γ antibody was added for a period of 30 minutes and then washed twice as described. Cells were labelled with any additional fluorochrome-conjugated primary antibodies at this stage if required. The cells were then resuspended in HBSS (300μl), stored at 4°C and analysed by flow cytometry within 12 hours.

Samples of fresh whole blood in ethylene diamine tetra-acetic acid (EDTA) anticoagulant were also used for direct antibody labelling in some instances. 10μL of fluorochrome-conjugated primary antibodies were added to 200μl of whole blood and incubated at room temperature in the dark for 15 minutes. 2mls of FACSLyse solution (BD) was subsequently added for 10 minutes at room temperature to lyse red blood cells. The cells were then centrifuged at 400g and resuspended in 500μl HBSS and this was repeated. The cells were then resuspended in HBSS (300μl) stored at 4°C and acquired within 12 hours.

All samples were acquired as list mode data using CellQuest software (Becton Dickinson Immunocytometry Systems) on a BD FACSCalibur flow cytometer in 4-colour mode. The number of events acquired was determined by the frequency of the events of interest within the cell pool. For experiments requiring quantification of rare events (defined as occurring with a frequency <5%) a minimum of 100 positive events were acquired wherever possible to give an intra assay co-efficient of variation (CV) of 10%. Data were subsequently analysed using CellQuest and CellQuest Pro software (BD). Data files for analysis were exported directly into Excel spreadsheets (Microsoft). Dot plot and histogram images for illustrative purposes were created using Windows Multiple Document Interface for Flow Cytometry software (WinMDI).

2.2.2 Basic Regions and Gates for Flow Cytometric Analysis

A forward scatter (FSC) versus (vs) side scatter (SSC) dot plot was always created for each list mode data file to be analysed on CellQuest/CellQuest Pro Software and an initial region (Region 1) drawn around the area of live lymphocytes.

To identify $CD3^+$ T cells a new dot plot (FSC vs CD3) was created gated on Region 1 (Gate 1) and a second region drawn around the $CD3^+$ cells (Region 2). For assessment of expression of a single antigen (e.g. CD69) on viable $CD3^+$ T cells a new dot plot (FSC vs CD69) was created gated on 'Region 1 and Region 2' (Gate 2). For assessment of expression of a single antigen (e.g. CD69) on subsets of $CD3^+$ cells (e.g. $CD3^+CD8^+$ cells) a new dot plot (FSC vs CD8) was created gated on Gate 2 and a region around the $CD8^+$ cells was drawn (Region 3). A new dot plot (FSC vs CD69) was created gated on 'Gate 2 and Region 3' (Gate 3).

For the assessment of antigen expression on live responder cells in co-culture with irradiated stimulator cells an initial FSC vs SSC dot plot was drawn and by 72 hours of co-culture a discrete region could be drawn around live responders with apoptotic (smaller) stimulator cells appearing to the left of the live responders.

When live cells responded to mitogenic stimuli (e.g. stimulatory anti-CD3 antibody OKT3, allostimulation) responder cells became activated lymphoblasts and enlarged, thus appearing to the right of the live lymphocyte region on a FSC vs SSC dot plot. Region 1 was expanded to include such responder cells in these instances. More complex and specific regions/gating procedures (including backgating) are described in individual chapters. The process of high-speed cell sorting on the FACSVantage device is described in Chapter 5.

2.2.3 MHC Class I-Peptide Tetramer Staining of $CD8^+$ T Cells

MHC Class I Peptide tetramers consist of 4 biotinylated HLA Class I molecules loaded with specific peptides (presented in conjunction with the HLA Class I molecule) coupled to a streptavidin molecule that has been conjugated to a fluorochrome (Figure 2.1). HLA A*0201 peptide tetramers conjugated with the PE fluorochrome (a gift from the Anthony Nolan Research Institute, and

Proimmune) were used to assess numbers of CMV-specific cytotoxic T lymphocytes.

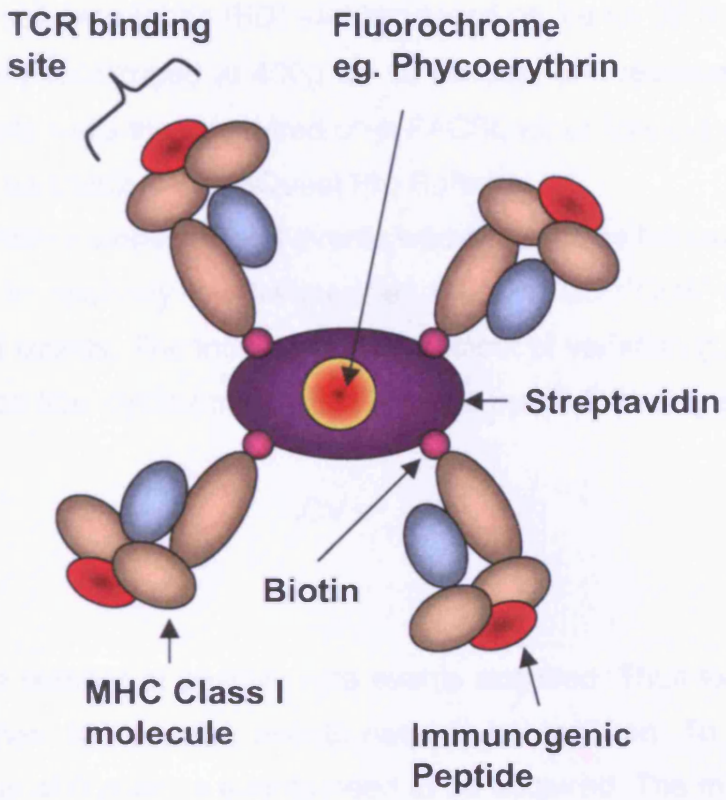

Figure 2.1 The molecular structure of an HLA Class I –peptide tetramer with the peptide-MHC specific TCR binding site indicated.

Tetramers were stored at 4° C in the dark. The optimal amount of tetramer added per million cells was titrated down to minimise non-specific binding. Generally 0.5-1ug of tetramer was added to 10^6 PBMCs along with 10μL of anti-CD3-APC antibody and 10μL of anti-CD8-FITC (or –PerCP) in complete medium in polystyrene tubes (BD) and incubated on ice for 30 minutes. The cell suspension was centrifuged at 400g for 10 minutes and resuspended in 300μL of HBSS. Events were then acquired on a FACSCalibur flow cytometer and data was analysed on CellQuest/CellQuest Pro Software.

No fixed minimum number of total events was acquired as tetramer binding cells usually occur in relatively low frequencies (<5% of $CD3^+CD8^+$ cells) and thus qualify as rare events. The intra-assay co-efficient of variation (CV) of such rare events acquired flow cytometrically is described by the following equation

$$CV = \frac{100}{\sqrt{n}}$$

where n is the number of positive rare events acquired. Thus to obtain a CV of <10% more than 100 positive events need to be acquired. To obtain a CV of <5% more than 400 positive events need to be acquired. The minimum number of positive events acquired, whenever possible was 100 to give a CV of 10% or less.

The calculation of tetramer frequency (as percentage of $CD3^+CD8^+$ cells) was done as follows: an initial FSC vs SSC dot plot was created and Region 1 created around the live lymphocytes. A new FSC-CD3 dot plot was created gated on Region 1. A new region (Region 2) was drawn around $CD3^+$ cells, and a new tetramer vs CD8 dot plot created gated on 'Region 1 and Region 2'. The percentage of tetramer$^+$ cells was then calculated using quadrant analysis. This strategy is illustrated in Figure 2.2.

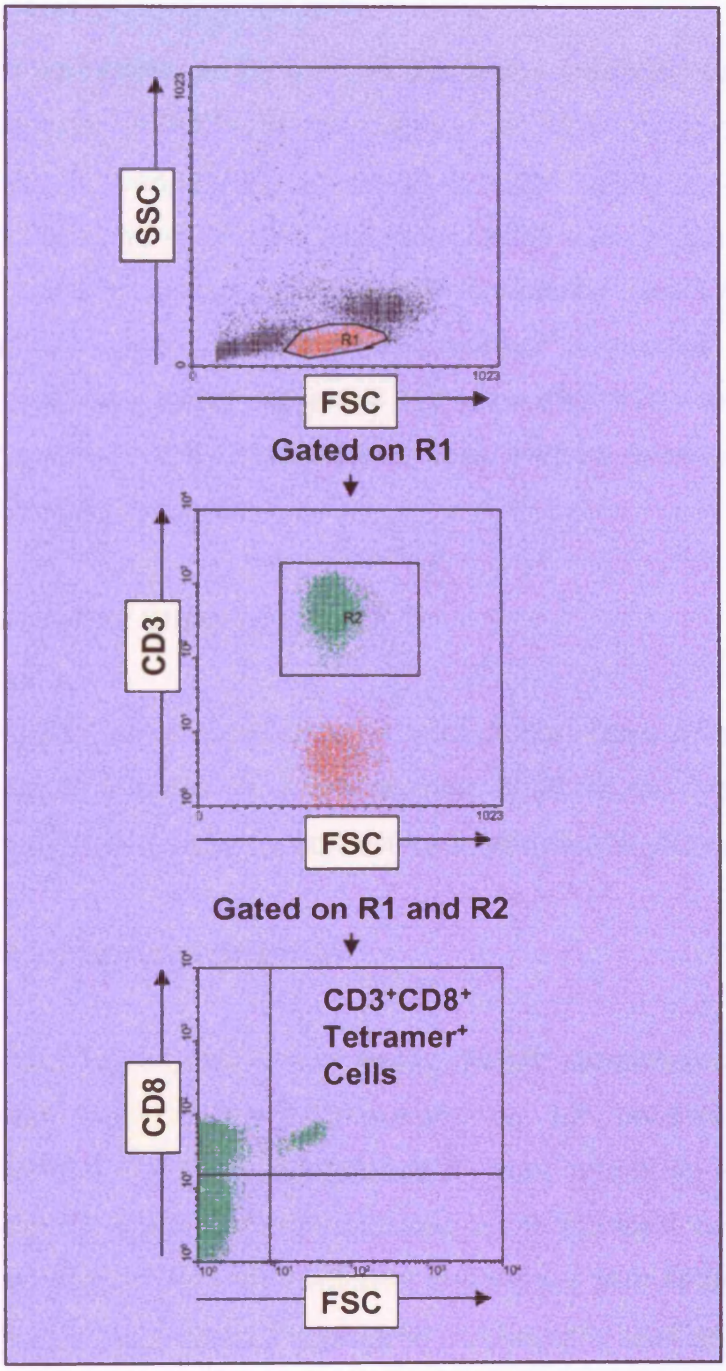

Figure 2.2 Regions and gates used to calculate the frequency of $CD3^{+}CD8^{+}$ Tetramer $^{+}$ cells. (From the CD8 vs FSC dot plot, percentage $CD3^{+}$ $CD8^{+}$ $Tetramer^{+}$ cells is the number of cells in the upper right quadrant/number of cells in upper right and upper left quadrants x 100%).

2.3 Allostimulation Techniques and Proliferation Assays

Allostimulation was achieved by the co-culture of γ-irradiated stimulator PBMCs with live responder PBMCs in the MLR. For HLA-mismatched stimulator-responder pairs (corresponding to recipient-donor pairs) allostimulation was achieved via co-culture in the standard MLR. For HLA-matched pairs allostimulation was achieved in the cytokine-modified and OKT3 pre-treated MLRs. MLRs were used as proliferation assays to quantify the alloreactive response by responder incorporation of thymidine after 120 hours of incubation, and also to generate $CD69^+$ alloreactive cells within a responder cell fraction prior to cell sorting procedures.

2.3.1 γ-Irradiation of Stimulator Cells

All stimulator cells were exposed to a γ-irradiation dose of approx 30Gy by irradiation for 6.5 minutes in a Gammacel 3000 ELAN irradiator (Nordion International Inc) which emits approx 500 rads per minute (5Gy per minute).

2.3.2 Mixed Lymphocyte Reaction

For the standard MLR proliferation assay, PBMC suspensions were used in complete media (with human AB serum) at 10^6 cells/ml. 10^5 irradiated stimulators/well and 10^5 live responders/well were added to round bottom 96 well plates (Nunc) in triplicate and incubated under standard conditions. Control wells were live responders with irradiated autologous stimulator cells. After 108 hours of incubation 1μCi tritiated thymidine (Amersham) was added to each well and after 120 hours of incubation plates were harvested on a plate harvester and counted on β-scintillation counter (Wallac) Proliferation of responders in response to allogeneic stimulus was expressed as mean counts per minute (cpm) above autologous control.

In order to generate $CD69^+$ alloreactive cells within a responder cell fraction prior to cell sorting procedures the mixed lymphocyte culture was set up on various larger scales in 24 well plates, $80cm^2$ cell culture flasks and Lifecell and Opticyte cell culture bags ('bulk MLRs'). Both irradiated stimulator and

responder PBMC cell concentrations were fixed at 10^6/ml and the stimulator responder ratio was (unless otherwise stated) fixed at 1:1. Bulk MLRs were depleted of $CD69^+$ responder cells after 72 hours of incubation.

2.3.3 The Cytokine-Modified MLR

In HLA-matched stimulator-responder samples the MLR was modified by the pre-treatment of stimulator cells with cytokines. Stimulator PBMCs were incubated at 10^6 cells/ml in complete medium (with human AB serum) for 24 hours under standard conditions with 1000iu/10^6 cells of recombinant human IFN-γ and recombinant human TNF-α (R &D systems). Cells were then centrifuged at 400g for 10 minutes and resuspended in complete medium and the wash step repeated prior to γ irradiation. Irradiated stimulator cells were then added to live responder cells under the conditions described in Chapter 2.3.2 with the addition of 1000iu/10^6 responder cells of recombinant human IL-4 (R &D) to the MLR. Negative control wells were cytokine pre-treated autologous irradiated stimulators and live responders, and positive controls were irradiated HLA-mismatched cytokine pre-treated third party stimulator cells and live responders (Third Party Controls).

The cytokine modified MLR was used both as a proliferation assay for HLA-matched stimulators/responders and as a bulk MLR technique prior to depletion of $CD69^+$ alloreactive cells. For the proliferation assay the results were expressed as mean counts per minute (cpm) (above autologous control) as an absolute value and also as a percentage of the equivalent result with the third party control (the Relative Response Index (RRI). A RRI of >/=5% was considered positive in the cytokine modified MLR.

2.3.4 The OKT3 Pre-Treated MLR

In HLA-matched stimulator-responder samples the MLR was also modified by the pre-treatment of the stimulator cells with the mitogenic stimulatory anti-CD3 antibody OKT3. OKT3 was obtained from a fermentation system using a murine hybridoma secreting human mitogenic OKT3 (American Type Culture Collection Cell Line CRL-8001, ATCC) The hybridoma was produced by the fusion of

P3x63Ag8UI mouse myeloma cells with splenocytes from CAF1 mice immunized with human peripheral blood lymphocytes and secretes murine IgG2a reactive to the human T cell CD3 molecule. The concentration of OKT3 in hybridoma supernatant was quantified by murine IgG Enzyme Linked Immunosorbent Assay (ELISA) (Bethyl laboratories Inc) After optimisation experiments 1ml of filtered hybridoma supernatant containing 500ng/ml of OKT3 was immobilised on a 24 well plate by overnight incubation at 4°C or 4 hours of incubation at 37°C. Wells were then washed twice with sterile HBSS and 10^6 stimulator PBMCs in 1ml of complete medium (with human AB serum) was added. The plates were incubated for 48 hours under standard conditions and the cells were then removed by pipette, diluted to a volume of 10mls in HBSS and centrifuged at 400g for 10 minutes. This washing step was repeated twice. The cells were resuspended at 10^6/ml and irradiated prior to use as stimulators in the MLR. No additional cytokines were added to the MLR. Negative control wells were autologous irradiated OKT3 pre-treated stimulators and positive controls were live responders with irradiated HLA-mismatched OKT3 pre-treated third party stimulators cells.

The OKT3 pre-treated MLR was used both as a proliferation assay for HLA-matched stimulators/responders and as a bulk MLR technique prior to depletion of $CD69^+$ alloreactive cells. For the proliferation assay the results were expressed as mean cpm (above autologous control) as an absolute value and also as a percentage of the equivalent result with the third party control (RRI).

2.4 The CD69 Selective Allodepletion Schema

Alloreactivity was determined before (primary MLR) and after (secondary MLR) selective depletion of $CD69^+$ alloreactive cells. Alloreactivity was quantified as responder cell proliferation in these MLRs with co-culture with the original irradiated stimulator cells (first party alloreactivity) or HLA-mismatched third party irradiated stimulator cells (third party alloreactivity)

2.4.1 HLA-Mismatched Stimulator-Responder Pairs

In the HLA-mismatched setting the MLR was used to assess first party alloreactivity using first party irradiated stimulators and unmanipulated responder cells (primary first party MLR) and CD69 depleted responder cells (secondary first party MLR)

Residual first party alloreactivity (%) was expressed as:

$$\frac{\text{Secondary first party MLR (cpm)-autologous control x100\%}}{\text{Primary first party MLR (cpm)-autologous control}}$$

The MLR was used to assess third party alloreactivity using third party irradiated stimulators and unmanipulated responder cells (primary third party MLR) and CD69 depleted responder cells (secondary third party MLR)

Residual third party alloreactivity (%) was expressed as:

$$\frac{\text{Secondary third party MLR (cpm)-autologous control x100\%}}{\text{Primary third party MLR (cpm)-autologous control}}$$

2.4.2 HLA-Matched Stimulator-Responder Pairs

In the HLA-matched stimulator-responder pairs the cytokine and OKT3 modified MLRs were used to potentiate allostimulation. Proliferative responses to first party stimulators in the primary and secondary MLRs were expressed as percentage of the response seen to third party stimulators (RRI). The CD69 allostimulation and selective allodepletion schema with assessment of alloresponses by MLR responder cell tritiated thymidine uptake at 120hrs is illustrated in Figure 2.3.

2.4.1 HLA-Mismatched Stimulator-Responder Pairs

In the HLA-mismatched setting the MLR was used to assess first party alloreactivity using first party irradiated stimulators and unmanipulated responder cells (primary first party MLR) and CD69 depleted responder cells (secondary first party MLR)

Residual first party alloreactivity (%) was expressed as:

$$\frac{\text{Secondary first party MLR (cpm)-autologous control x100\%}}{\text{Primary first party MLR (cpm)-autologous control}}$$

The MLR was used to assess third party alloreactivity using third party irradiated stimulators and unmanipulated responder cells (primary third party MLR) and CD69 depleted responder cells (secondary third party MLR)

Residual third party alloreactivity (%) was expressed as:

$$\frac{\text{Secondary third party MLR (cpm)-autologous control x100\%}}{\text{Primary third party MLR (cpm)-autologous control}}$$

2.4.2 HLA-Matched Stimulator-Responder Pairs

In the HLA-matched stimulator-responder pairs the cytokine and OKT3 modified MLRs were used to potentiate allostimulation. Proliferative responses to first party stimulators in the primary and secondary MLRs were expressed as percentage of the response seen to third party stimulators (RRI). The CD69 allostimulation and selective allodepletion schema with assessment of alloresponses by MLR responder cell tritiated thymidine uptake at 120hrs is illustrated in Figure 2.3.

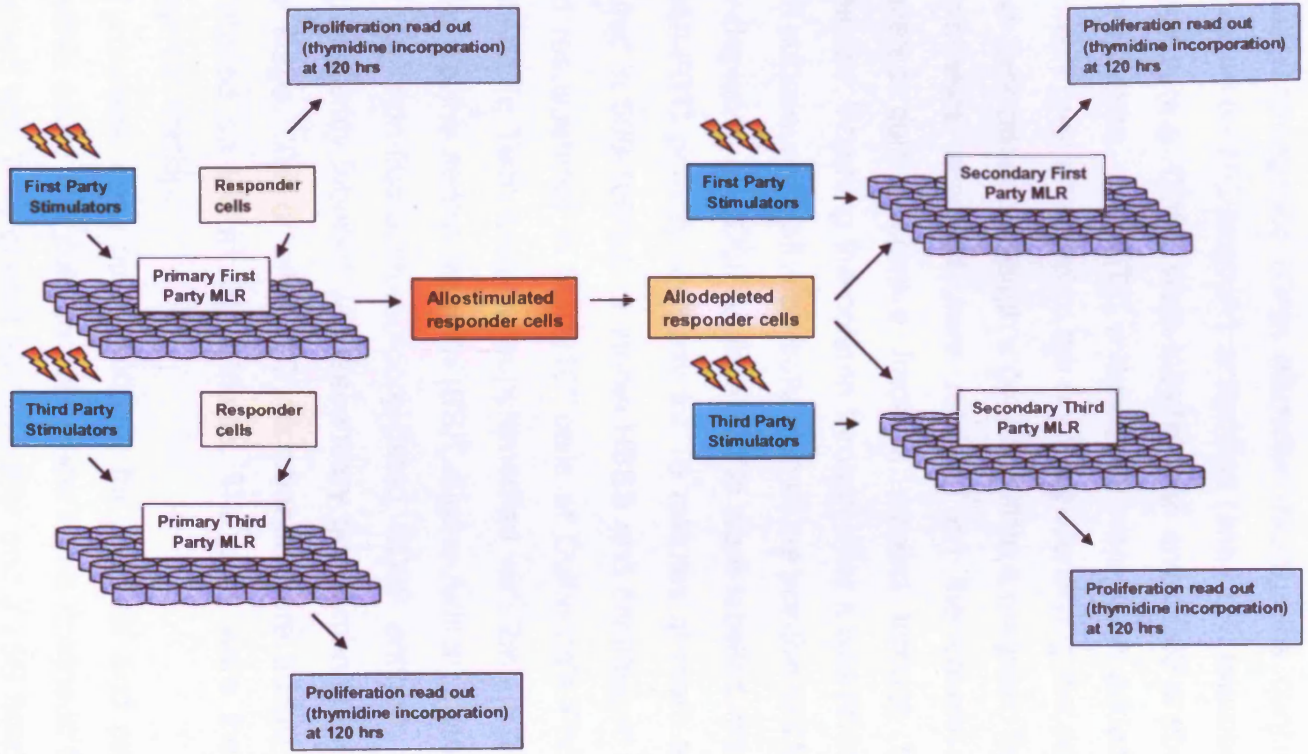

Figure 2.3 Schema for allostimulation and selective allodepletion of $CD69^+$ cells and assessment of alloreactivity (pre- and post-depletion) to first and third party stimulators. Note that in the HLA-matched setting both the first and third party stimulators were pre-treated with either cytokines or stimulatory OKT3.

2.5 Cell Sorting Techniques

2.5.1 MACs Immunomagnetic Depletion

This is a sterile cell sorting technology (Miltenyi Biotech) that utilises colloidal super-paramagnetic 50nm diameter microbeads conjugated with monoclonal mouse anti-FITC Isomer-1 antibodies (anti-FITC microbeads). Cells expressing antigens (e.g. CD69) were labelled with anti-CD69 antibody conjugated to FITC fluorochrome. Anti-FITC microbeads were then added, binding to the FITC fluorochrome attached to the antibody identifying the cells to be isolated. Cells were then passed through a column within a magnetic field. Cells with anti-FITC microbeads attached were retained on the column magnetically and the depleted cells (negative fraction) passed through the column and were collected. Washing the column through after it was removed from the magnetic field subsequently allowed collection of the positive cell fraction.

For depletion of $CD69^+$ cells, PBMCs were labelled with $10\mu L/10^6$ total cells of CD69-FITC primary antibody for 15 minutes at room temperature. Cells were diluted in 500-1000µL of sterile HBSS and centrifuged at 400g for 10 minutes and resuspended in $90\mu L/10^6$ cells of Dulbecco's Phosphate buffered saline (Gibco/Life Technologies) supplemented with 2mM EDTA (Sigma-Aldrich) and 0.5% bovine serum albumin (BSA, Sigma-Aldrich) ('MACS Buffer') at 4°C. If a primary non-fluorochrome-conjugated CD69 antibody was used, cells were subsequently labelled with secondary goat anti-mouse FITC antibody (BD) at this stage. 10µl of anti-FITC microbeads were added per 10^7 total cells and incubated on ice for 20 minutes. The cells were then ready for immediate magnetic sorting.

AS columns were used, suitable for removal and retention of up to $3x10^7$ positive cells. The column was place in the magnetic field of the VarioMACS, prefilled with 2mls of cold MACS buffer and a 25G flow resistor attached to the outflow. The cold-labelled cell suspension was applied to the column in situ and flowed through the column with a flow rate of 0.3ml/minute. The negative cell fraction was collected from the outflow. The column was washed in situ with 3mls of MACS buffer.

The column was removed from the VarioMACS magnetic field and the positive cell fraction collected by back flushing through of the column under pressure with 3mls cold MACS buffer.

2.5.2 Dynabead Immunomagnetic Depletion

Dynabeads (Dynal Biotech) are paramagnetic polystyrene beads with affinity purified sheep anti-mouse IgG antibody covalently bound to the surface.
The labelling of cells with a primary murine antibody and subsequent labelling with the Dynabead enables cell sorting by application of a magnetic field which retains cells bound to Dynabeads. The system may be used as part of a sterile cell selection technology at both a small laboratory scale using a hand-held magnet or at a larger clinical scale using the Isolex 300i automated cell sorting device.
Dynabeads were supplied in a sterile, non-pyrogenic suspension of 10mls containing $4x10^8$ beads/ml in PBS with 0.1% human serum albumin. Dynabeads were prepared by washing in 10mls of sterile PBS and application to a hand-held magnetic particle concentrator (MPC) for 1 minute. The wash solution was drained off and the beads resuspended at a concentration of $4x10^8$ beads/ml in PBS. For small-scale $CD69^+$ cell depletions, PBMC cell suspensions were incubated with non-fluorochrome-conjugated murine anti-CD69 antibody at optimal concentration (individually determined for each antibody used) for 15 minutes at room temperature. Cells were diluted in 500µL of sterile HBSS, centrifuged at 400g for 10 minutes and resuspended in sterile HBSS to a concentration of 10^6/ml in HBSS. Washed and thoroughly mixed Dynabeads were added to 10^6 labelled cells at various bead:cell ratios and incubated at room temperature in 2ml polystyrene tubes (Sarstedt) for 30 minutes with continuous vertical rotation. The tubes were then placed on to the MPC for 1 minute and the supernatant (containing the negative cell fraction) removed carefully by Pasteur pipette.
For clinical scale up procedures the Isolex 300i automated device (with 2.5i software) (Nexell) was used to immunomagnetically deplete large numbers of CD69 labelled cells with Dynabeads. The procedure is described in detail in Chapter 7.

2.5.3 Eligix Particle Immunomagnetic Depletion

Goat anti-mouse (GAM) High Density Microbeads (HDM) (Eligix) are Inco 123 Nickel particles about 10μm in diameter coated with GAM IgG antibody. Mouse IgG can be rapidly coupled to these HDMs. The GAM is specific to Fc-γ and therefore must always be used with whole mouse IgG (not Fab or $F(ab')_2$ fragments. For small-scale laboratory depletion of $CD69^+$ cells GAM-HDM particles were reconstituted by washing with in 10mls of HBSS twice using the MPC to retain the particles. Particles were then reconstituted with 10 mls 2% dextran (Sigma). 100μL of reconstituted particle suspension contained 25 x 10^6 GAM-HDM particles. 100μl of reconstituted GAM-HDM particle suspension were added to 2ml microcentrifuge tubes (Sarstedt) containing 1.5mls Phosphate-Buffered Saline (PBS, Gibco-Life/Technologies). The tube was placed onto the MPC to retain the particles, the supernatant decanted and discarded, and 1.5mls of PBS added to the tube. This was repeated twice to leave a wet GAM-HDM pellet. An optimum concentration of primary murine CD69 antibody was added in 100μL volume in PBS with 0.5% BSA, thoroughly mixed and incubated at room temperature for 15 minutes, mixing occasionally. The GAM-HDM particles were retained on the magnet and washed with 1.5mls HBSS three times and the particle degaussed using a hand-held degaussing device (Eligix). 1ml of PBMCs (10^6 cells) were then added to the labelled GAM-HDM particles and mixed with continuous vertical rotation for 15 minutes at room temperature. The HDM-GAM particles were then allowed to settle with gravity upright for 10 minutes and the tube applied to the MPC. The depleted cell fraction was collected in the supernatant by Pasteur pipette.

2.5.4 Cell Sorting Parameters

The frequency of $CD69^+$ cells was determined by labelling with fluorochrome-conjugated monoclonal antibodies and flow cytometric analysis.

Depletion efficiency of $CD69^+$ cells was defined as:

$$\frac{(\%CD69^+ \text{ cells prior to sorting}) - (\%\ CD69^+ \text{ cells after sorting}) \times 100\%}{(\%CD69^+ \text{ cells prior to sorting})}$$

Depletion efficiency of $CD69^+$ positive cells was calculated in this way for $CD3^+$, $CD3^+CD4^+$ and $CD3^+CD8^+$ cell subsets, identified flow cytometrically.

Cell yield of $CD69^-$ cells was defined as:

$$\frac{\text{Number of } CD69^- \text{ cells after sort} \times 100\%}{\text{Number of } CD69^- \text{ cells before sort}}$$

2.6 IFN-γ ELISpot Assay

The IFN-γ Enzyme Linked Immunospot (ELISpot) assay (MabTech) was used to assess cellular responses specific to a range of immunogenic CMV-peptides and to an EBV-peptide. The MabTech kit using polyvinylidene difluoride-lined (PVDF) plates was chosen as it has low intra- and interassay variance.
PVDF-lined 96 well plates (Millipore) were pre-wet with 100µl/well 70% ethanol for 10 minutes at room temperature and then washed 3 times with 200µl of sterile filtered PBS by pipette.
Coating antibody (Murine IgG1 anti-human IFN-γ antibody 1-DIK) was diluted to 15µg/ml in sterile filtered PBS and 100µl of working dilution added to each well of a prepared PVDF 96 well plate, which was then incubated overnight at 4°C.
The plate was then washed by hand six times with 200µl sterile PBS/well to remove non-bound coating antibody. 150µl of complete media was added to each well for 1 hour at 37°C 5% CO_2 60% humidity (to block further non-specific antibody binding) and decanted prior to addition of cells.

Fresh or recently resuscitated PBMCs at 10^6/ml in complete media were added to the wells prior to the addition of stimulatory peptide. Cells were added to wells at 5 x 10^4, 1 x 10^5 and 2 x 10^5 cells/well. Immunogenic CMV- or EBV-peptides (ProImmune) with known HLA restriction were stored dissolved in DMSO at high concentration at –70°C (2mg peptide in 200µl DMSO) in 5µl aliquots. The working concentration of peptide was 10 µmolar i.e. 1:1000 dilution of frozen stock. This diluted out the DMSO so minimal toxicity to cells would result. Peptides were reconstituted in complete medium (with human AB serum) to 1µg/100µL for use in the ELISpot assay. 1µg of CMV- or EBV-peptide of appropriate HLA Class I restriction (in 100µl complete medium) per 10^5 cells was added to each well and incubated for 20 hours. Negative control wells were cells without CMV- or EBV-peptide and cells with CMV-peptide restricted to an HLA Class I type not possessed by the cells ('dummy peptide'). Positive controls were 10^5 cells plus 10uL phytohaemagglutin (PHA, Sigma-Aldrich). Plates were incubated under standard conditions for 20 hours. Cells were then removed by washing six times with 200µl sterile PBS. A second biotinylated murine IgG1 anti-human IFN-γ antibody (7-B61), (which recognises a different epitope of human IFN-γ to the 1-DIK antibody), was then diluted 1:1000 in filtered sterile PBS with 0.5% FCS and 100µl was added to each well and incubated for 90 minutes at room temperature. Wells were then washed six times with sterile, filtered PBS. Streptavidin-Alkaline Phosphatase was diluted 1 in 1000 in filtered PBS with 0.5% FCS and 100µl was added to each well and incubated for 1 hour at room temperature. Wells were washed 6 times with sterile filtered PBS and 100 µl of reconstituted alkaline phosphatase colour change substrate (Biorad) added per well. Plates were agitated gently at room temperature for 10-20 minutes until spots appeared just visible to the naked eye. Colour development was then stopped by washing three times with 200µl/well of tap water. Plates were left to air dry at room temperature overnight (during which time the spots became more prominent) and wells were subsequently photographed using an digital camera (Olympus) and dissection microscope. The ELISpot assay is depicted in Figure 2.4

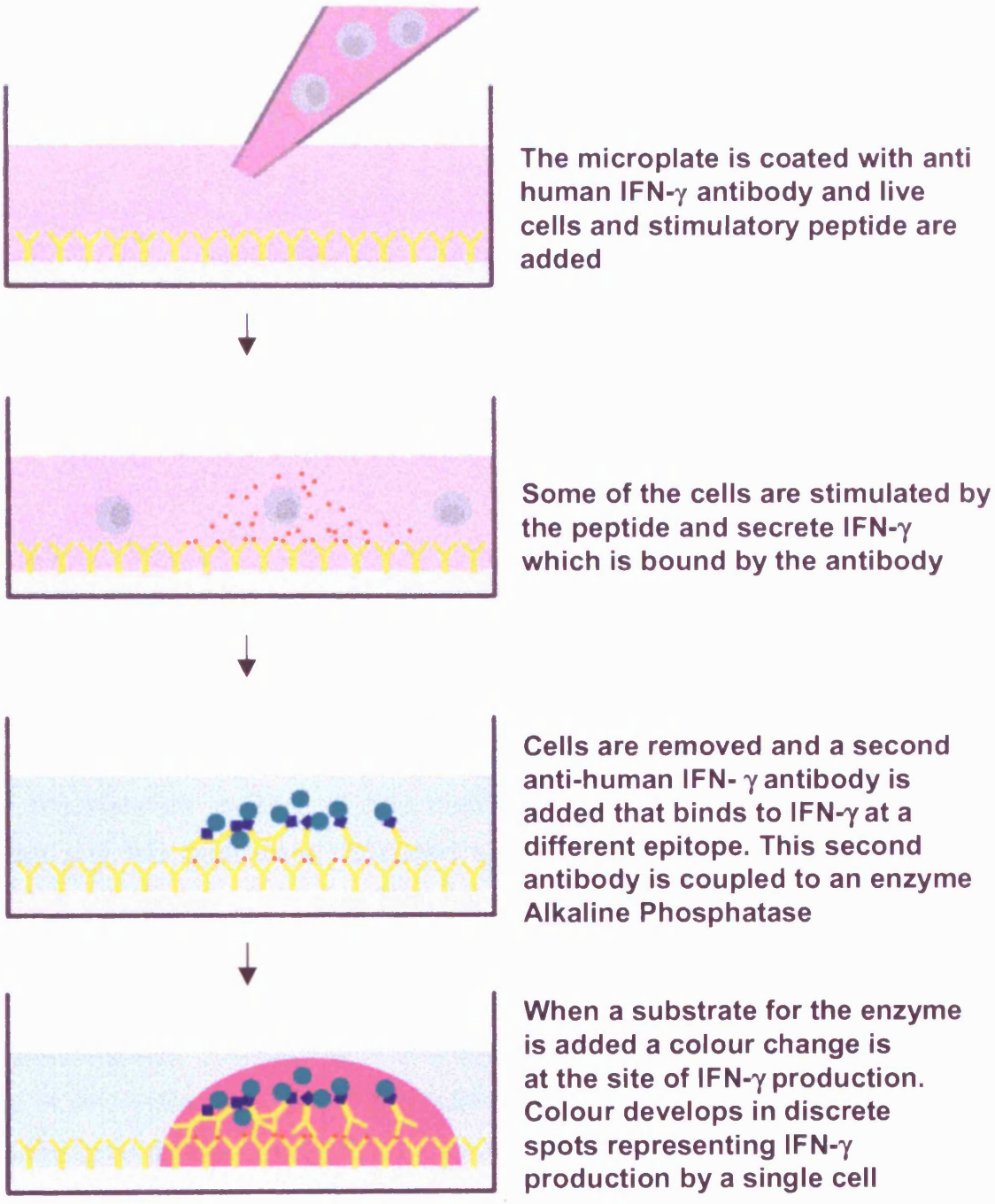

Figure 2.4 The principle of the IFN-γ ELISpot Assay.

Frequency of responding cells was defined as:

$$\frac{\text{Mean number of dots/well in test wells} - \text{number of dots/well (negative controls)}}{\text{Number of cells/well}}$$

Frequency of CMV- or EBV-specific cells (percentage of $CD3^+CD8^+$ cells) was defined as (percentage $CD3^+CD8^+$ PBMCs x frequency of responding cells). Note that the percentage of $CD3^+CD8^+$ within PBMC cells was determined by flow cytometry.

2.7 IFN-γ Secretion Assay

The APC-conjugated flow cytometric intracellular IFN-γ cytokine secretion assay detection kit was used (Miltenyi).

PBMCs were stimulated with 5ug CMV-peptide/10^6 cells in polystyrene tubes (Kendall-Elkay) for 12 hours at 37°C. Up to 10^6 PBMCs were suspended in 1 ml of cold MACS buffer, centrifuged at 400g for 10 minutes at room temperature and resuspended in 90μL of cold medium. 10μl of IFN-γ catch reagent was added and the cells were incubated on ice for 5 minutes. Warm complete medium was added to the cells (1ml per 10^6) which were then incubated for 45 minutes at 37° C with intermittent agitation to resuspend settled cells. The cells were then put on ice and centrifuged at 400g for 10 minutes at room temperature after adding 3mls of cold buffer. The cell pellet was resuspended in 90μl of cold buffer and 10μl of IFN-γ detection antibody (APC-conjugated) was added along with 5μl of CD3-FITC, CD4-PE, and CD8-PerCP (all BD). The cells were incubated on ice for 10 minutes and then 2mls of cold buffer added and centrifuged at 400g for 10 minutes at room temperature. The cell pellet was resuspended in FACSFlow and samples acquired on a flow cytometer. Negative controls were cells stimulated with no peptide or with a dummy peptide. Note that this protocol is optimised for <5% cytokine secreting cells.

2.8 Murine Anti-human IgG ELISA

Murine anti-human IgG was quantified by ELISA in hybridoma supernatants using an ELISA kit (Bethyl).

Affinity purified capture antibody (supplied at 10µg/ml) was diluted 1/100 with coating buffer (0.05M Sodium Carbonate pH 9.6). 100µl was added to each well of 96 well Immulon plates (Nunc). The plate was incubated overnight under standard conditions. Capture antibody was removed from wells by aspiration and each well carefully washed with 200µl of wash solution (50mM Tris, 0.14M NaCl, 0.05% Tween 20, pH 8.0) 3 times. 200µl of post-coat solution (50mM Tris, 0.14M NaCl, 1% BSA, pH 8.0) (all Sigma-Aldrich) was added to each well and incubated for 30 minutes. After incubation each well was washed as above 3 times. Standards and test samples were diluted in sample diluents (dilutions were based on the expected concentration of the analyte to fall within the concentration range of the standards. Standard mouse reference serum was supplied with a concentration of murine IgG of 7.3mg/ml and was diluted to 500ng/ml by the addition of 100µL of standard to 730µL of sample diluent (50mM Tris, 0.14 M NaCl, 1% BSA, 0.05% Tween 20, pH 8.0) to yield 10,000ng/ml and then 100µL of this added to 1.9ml of sample diluent. Serial 2-fold dilutions were then made for standards between 250 and 7.8 ng/ml. 100µl of standard or test sample was added to duplicate wells and incubated for 30 minutes at room temperature. After incubation the wells were washed 4 times. 100µl of diluted goat anti-mouse IgG Fc-Horseradish peroxidase-conjugated antibody was added to each well and incubated for 60 minutes at room temperature. This was followed by 4 wash cycles. The prepared enzyme substrate colour change reagent (Amersham) was added at 100µl/well and incubated for 20 minutes at room temperature. 100µL of stop reaction reagent (Amersham) was then added to each well. A microtitre plate reader (Anthos Labtec) was then used to assess the light absorbance at 450nm (appropriate for the colour change substrate used.) A standard curve was created and the mean concentration (of two readings) of murine IgG from each test sample calculated.

2.9 PKH-26 Dye Labelling of Cells

Target cells were cell surface membrane labelled with a fluorescent cell linker kit, which incorporates the fluorescent dye PKH-26 (Sigma-Aldrich). PKH-26 is a red fluorochrome with emission wavelength range homology with PE, which fluoresces very brightly with an emission peak of 580nm and can thus be detected in the FL2 channel on a FACSCalibur flow cytometer. This allows PKH-26 labelled cells to be identified by flow cytometric analysis. High-level fluorescence with PKH-26 staining, membrane dye stability and lack of dye transfer to effector cells has been documented.[Lowdell *et al.* 2002]

To label with PKH-26 dye, cells were centrifuged at 200g for 10 minutes, counted and viability assessed by trypan blue staining, and resuspended in 1ml of labelling diluent at a concentration of $4x10^6$ cells/ml. 4μL of PKH-26 membrane dye was then added to 1ml of labelling diluent in a separate microcentrifuge tube. 0.5ml of diluted dye was added to 0.5ml of target cells and incubated on ice for 2 minutes. After 2 minutes, 0.5mls of neat FCS was added to the cell suspension to stop the reaction. The labelled cells were then washed twice in a micro-centrifuge. Adequate cell labelling was confirmed by analysis of cells on a flow cytometer. Labelled cells were then used variously in the MLR or as targets in cell-mediated killing assays.

2.10 PKH-26 Dye Cytotoxicity Assay

Briefly, T2 target cells (a kind gift from the Anthony Nolan Research Institute) were split in culture 24 hours prior to the assay to ensure that they were in exponential log phase.

Target cells were untreated T2 cells suspended in complete medium at 10^6 cells/ml or T2 cells to which excess CMV NLV peptide (10 μg/10^6 cells) had been added, incubated for 12 hours under standard conditions, centrifuged for 10 minutes at 200g and resuspended in complete medium.

Effectors were healthy CMV IgG positive HLA A*0201$^+$ donor CTLs, either unselected or CMV-selected (after 3 rounds of stimulation with CMV whole antigen (Dade Behring) at day 1 (1mg/ml) and 0.5mg/ml at days 7,and 14), (supplied by Dr Kwee Yong).

Target cells were labelled with PKH-26 as described in Chapter 2.14. The PKH-26 killing assay was then set up (with each condition in duplicate) with an effector: target cell ratio of 10:1. A minimum of $2x10^5$ PKH-26 labelled target cells were added to effector cells and gently centrifuged for 1 minute to pellet the cells. The assay was incubated for 4 hours under standard conditions. After incubation, 100μL of Propidium Iodide (1μL/ml) was added to each tube and cells analysed by flow cytometry. PKH-26-labelled target cells were identified by their intense fluorescence in the FL2 channel on the FACSCalibur flow cytometer and the percentage lysis of target cells was determined as the percentage of labelled target cells taking up the propidium iodide (PI), (which fluoresces in the FL3 channel on the FACSCalibur flow cytometer). Spectral overlap between PKH-26 and PI was eliminated electronically using appropriate controls. Background target cell death was determined in PKH-26 labelled target cells incubated under similar conditions in the absence of effector cells. Specific target cell killing was calculated as the (percentage target cell death – percentage background target cell death) for each condition. (Figure 2.5)

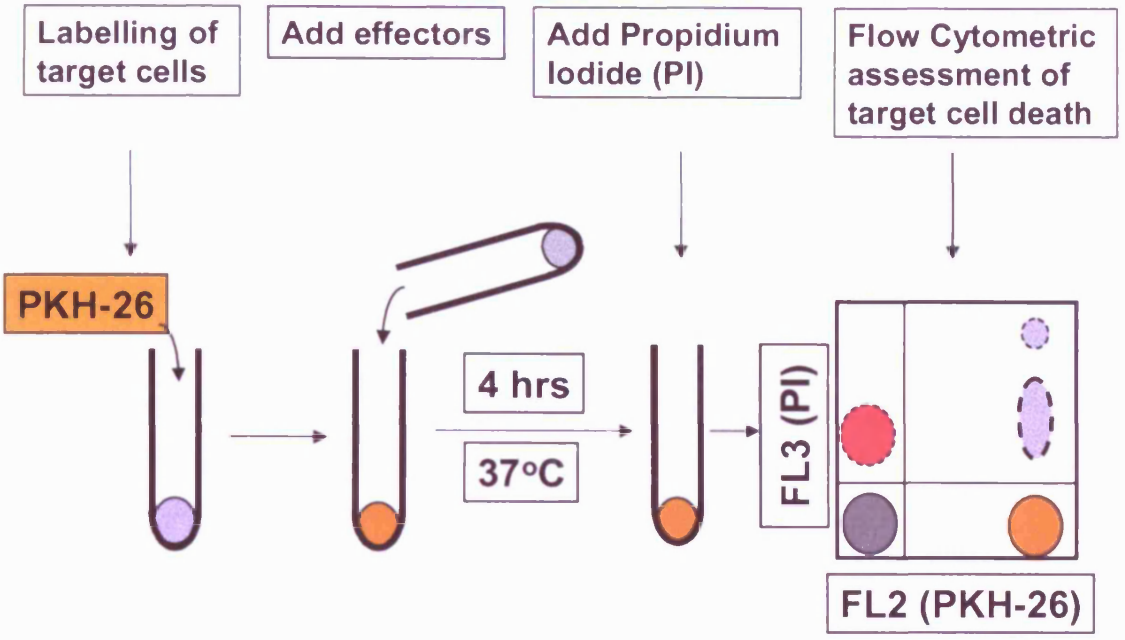

Figure 2.5 The principle of the PKH-26 dye release cytotoxicity assay. Target cells are labelled with PKH-26 prior to incubation with effector cells for 4 hours under standard conditions. Propidium iodide (PI) is the added to the cell mixture. The percentage target cell death can then be determined by flow cytometric analysis as the percentage of PKH-26^{bright} target cells also positive for PI (Cell frequency in upper right quadrant/cell frequency in lower right quadrant) x 100% on a FL2 vs. FL3 plot on a FACSCalibur flow cytometer. Specific target cell killing was calculated as the (percentage target cell death – percentage background target cell death).

2.11 T2 Cell Peptide Binding Assay

The ability of synthetic CMV-peptides to stabilize MHC Class I molecules on the surface of the T2 cell line was measured by flow cytometry. T2 cells only express HLA A*0201 Class I molecules on their surface and have weak expression due to their lack of the transporter-associated protein (TAP) and consequent inability to process antigenic peptide which is needed to stabilise the expression of HLA Class I molecules on the cell surface. Addition of exogenous HLA A*0201 binding peptide leads to stabilisation of HLA Class I molecules and consequently an increase in HLA Class I median channel fluorescence intensity assessed by flow cytometry.

T2 cells were split in culture 24 hours prior to the assay to ensure that they were in exponential log growth phase. CMV-peptide was added in excess (10 μg/10^6 cells) and the cells incubated overnight under standard conditions. 10^5 cells were diluted in 500μL HBSS and centrifuged for 10 minutes at 400g to wash them free of unbound peptide. Cells were then labelled with 10μL of W162 anti-HLA Class I FITC-conjugated antibody (Pharmingen) and acquired on a FACSCalibur flow cytometer (BD). Controls were cells with no peptide and a dummy (non HLA A*0201 restricted) CMV-peptide. The median channel fluorescence of cells was determined. The MHC-stabilization efficiency (MSE) for each peptide was calculated as the percent increase of the median channel fluorescence above that of the negative controls. Peptides with MSE greater than 50% were considered as positive binders.

2.12 HLA Typing

HLA typing was undertaken at the Anthony Nolan Research Institute for all normal control individuals and donor-recipient transplant pairs. HLA typing was performed using the Sequence-Specific Oligo-Primer (SSOP) technique for allelic typing at both class I and Class II antigens. Individual details of HLA types of samples used are given in the relevant chapters.

2.13 Consent

Written informed consent was given by all individuals concerned for the use of their blood samples for research purposes including cryopreservation and storage of cells for indefinite periods of time prior to experimental use. Unrelated donors gave written informed consent for use of their blood samples whilst at their local Transplant Centres before stem cell donation. Such samples were shipped to our laboratory in an anonymised form.

2.14 Statistical Analyses

The paired or unpaired two-tailed Student's t-test was used when samples exhibited similar variance. If a significant difference in sample variance was seen then Welch's correction was applied to the Student's t-test. A p value of <0.05 was chosen as the level for rejection of the null hypothesis.

Chapter 3 OKT3 Pre-treatment of Stimulatory Cells and Selective Allodepletion

3.1 Introduction

All modalities of selective alloreactive cell depletion rely on the principle of stimulation of alloreactive cells in a live responder (donor) cell population by irradiated stimulator (recipient) cells in a unidirectional MLR. The optimum number and type of stimulator cells remains to be determined and may vary depending upon the individual technique used, the degree of major and minor histocompatability disparity between stimulator and responder cells and the lineage of the cells to which a retained GvL effect is desired. It is important that the stimulator cells be free of malignant cells to prevent presentation of leukaemia-specific antigens and subsequent activation and depletion of responder cells with specific anti-leukaemic activity. The remission status of potential recipients of selectively allodepleted grafts should be confirmed using the most sensitive techniques available (which include the use of the polymerase chain reaction (PCR) for TCR or Immunoglobulin Heavy Chain gene rearrangements for lymphoid malignancies, known specific chromosomal translocations in myeloid malignancies, and/or patient-specific multicolour flow cytometric profiling).

Unselected PBMCs have been used in the unmodified MLR prior to selective allodepletion based upon CD25 expression in the haploidentical setting,[Cavazzana-Calvo *et al.* 1990;Cavazzana-Calvo *et al.* 1994] and CD69 expression in HLA-mismatched pairs in vitro.[Koh *et al.* 1999] Dendritic cell enriched stimulators (via a fibroblast monolayer) have been utilised for stimulator cells prior to allodepletion based on either CD25 or CD69 expression and on both.[Fehse *et al.* 2000a;Fehse *et al.* 2000b] Allostimulation prior to selective allodepletion based on CD25 expression in haplotypical mismatched stimulator-responder pairs has been found to be more reproducible in vitro with the use of EBV transformed LBCs derived from recipient stimulator cells,[Amrolia *et al.* 2003a] and this strategy has been utilized in a clinical pilot study of haploidentical paediatric stem cell transplants.[Amrolia *et al.* 2003b]

For selective depletion of alloreactive cells based on responder cell expression of activation antigens a measurable upregulation of activation antigen is

required for the immunomagnetic sorting of alloreactive cells and the assessment of the efficiency of this process, whereas an additional readout (e.g. responder cell proliferation in the MLR) is required for assessment of the selective abrogation of T cell responses to first party stimulators with retention of responses to third party stimulators. In the HLA A, B and DR-matched setting hTLP frequencies are often low and proliferative responses may not be seen.[DeGast *et al.* 1992;Dickinson *et al.* 1998]

Bishara et al reported a modified MLR in a series of HLA A, B, DR and DQ-matched (by serological typing) sibling pairs with pre-treatment of stimulator cells with IFN-γ and TNF-α prior to irradiation and the subsequent addition of IL-4 to the MLR.[Bishara *et al.* 1994] They defined a positive read-out as a proliferative response with HLA-matched stimulator PBMCs at 120 hours of co-culture of >/= 5% of the proliferative response seen with HLA-mismatched third party PBMC stimulator cells (the Relative Response Index, RRI). RRI was positive in 15 of 40 (38%) HLA-matched sibling pairs they tested. They found that the modified MLR had a positive predictive value of 66% for acute GvHD (all grades) in recipients of T cell depleted transplants and 70% for recipients of non T cell depleted transplants. The negative predictive value for development of acute GvHD was 93% and 90% respectively. The authors did not attempt to correlate the RRI in the modified MLR with the severity of acute GvHD. Use of an attenuated cytokine modified MLR (with no pre-treatment of stimulators, IL-4 added to the MLR culture only) has subsequently been reported in HLA A, B, and DR locus matched unrelated pairs. The RRI was >/=5% in 19 of 35 (54%) pairs tested and correlated with HLA C-mismatch.[Bishara *et al.* 1999]

The full cytokine-modified MLR has been utilized for CD69 allostimulation and depletion in vitro by Koh et al. He reported 5 of 9 HLA A, B and DR-matched (A and B serologically typed, DR antigens typed at allelic level) sibling and unrelated donor-recipient pairs tested had a positive cytokine mMLR (RRI>5%).[Koh *et al.*1999] Another group has recently used this strategy with data published in abstract form and the proportion of pairs RRI>/=5% was not stated.[Schumm *et al.* 2003] Thus the use of the cytokine-modified MLR as part of a CD69 selective allodepletion strategy is limited to the 40% of HLA A, B and DR-matched pairs that exhibit a RRI>/=5%.

The use of dendritic cell-enriched stimulator cells has the advantage of enriching the stimulator cell population with professional APCells that might be

more effective at presenting alloantigen, however myeloid-restricted antigen responses might be lost which might reduce the GvL effect if these antigens are not shared (or are differentially expressed) between normal host haematopoietic cells and myeloid leukaemia cells.

The use of EBV-LBC lines is not ideal for use in the HLA-matched setting due to the theoretical risk of loss of responder cell activity to EBV antigens presented by such stimulator cells and also has the disadvantage of possible direct transfer of the EBV genome during the graft engineering process, both of which could increase the risk of EBV-related post-transplant lymphoproliferative disorder (PTLD) (although PTLD was not evident in the single report of a clinical trial using this strategy).[Amrolia *et al* 2003b]

The demonstration of limited tissue distribution of mHags, specifically the limitation of expression of the HA-1 and HA-2 antigens to haematopoietic cells has led to the successful development of the strategy of harnessing allogeneic CTLs specific for these mHags to exert a specific GvL effect in the setting of haematopoietic malignancies.[de Bueger *et al.* 1992;Kloosterboer *et al.* 2004;Marijt *et al.* 2003;Mutis *et al.* 1999b] Although mHags with different levels of expression on myeloid and lymphoid lineage cells have yet to be identified, the antigen-presenting cell used for allostimulation prior to selective allodepletion in the HLA-matched setting would be of crucial importance if such lineage-restricted mHags exist.

The cytokine pre-treatment of stimulator cells with TNF-α, IFN-γ and /or IL-4 leads to selective upregulation of adhesion molecules (CD11a, CD54), HLA Class I and II molecules and co-stimulatory molecules (CD80, CD86) on $CD14^+$ monocytes with less effect on $CD3^+$ T cells.[Davies *et al.* 2003] This strategy may therefore preferentially increase presentation of myeloid/monocytes-restricted mHags in the HLA-matched setting and might also lead to loss of myeloid-restricted tumour antigen responses if used in a setting of myeloid-lineage malignancy.

The use of predominantly lymphoid stimulators could lead to the preferential presentation of lymphoid–restricted or lymphoid–specific antigens. This would have the theoretical advantage of preservation of responses to myeloid restricted antigens which might result in better anti leukaemic activity in the graft in the case of myeloid malignancy, but has the disadvantage of the use of non-professional APCells which might be less effective at presenting a broad

spectrum of alloantigens (particularly those presented in the context of HLA Class II molecules) required for effective stimulation (and subsequent removal) of alloreactive cells and abrogation of alloreactive responses.

When activated, human T cells express MHC class II antigens and adhesion molecules characteristic of APCells. Recent in vitro and in vivo evidence supports an antigen-presenting function for T cells. Human T cell clones have been shown to be capable of presenting influenza peptide antigens directly but not an appropriate strain of inactivated whole influenza virus, indicating an inability to process antigen conventionally.[Hewitt and Feldmann 1989] Antigen presentation by T cells (T-T presentation) induces an initial T cell activation phase as measured by proliferation and IL-2 production. These activated T cells became anergic only upon antigenic restimulation by professional APCells, as shown by a failure to proliferate or produce IL-2 or IFN-γ.[Taams *et al.* 1999] In this guise, T cells may provide down-regulatory signals for the immune response by inducing anergy in T cells that have already been activated. It has been suggested that this may represent an important negative mechanism for T cell homeostasis. [Pichler and Wyss-Coray 1994]

The ability of HLA-DR-expressing human T cell clones to function as APCells has been investigated using highly purified T cells. These T cell clones were unable to act as autonomous APCells, and recognition of nominal or alloantigens on the surface of T cells led to a state of non-responsiveness. One reason for this may be the relatively low expression of co-stimulatory molecules on T cells as MHC/TCR interaction without the second signal provided by ligation of co-stimulatory receptors is known to induce a state of anergy in responding T cells. In the presence of soluble peptide and PBMCs T cells were capable of displaying antigen to each other, and PBMC derived APCells provided co-stimulatory signals. [Sidhu *et al.* 1992]

A potential technique to potentiate antigen presentation by a PBMC pool is to pre-treat T cells within the stimulator PBMC pool with a stimulatory anti-CD3 antibody to activate T cells and potentially to increase their capacity to present alloantigens either directly or indirectly via non-T cells in the stimulator cell pool. This technique has recently been described to quantitatively expand the stimulatory cell pool prior to CD25-mediated selective allodepletion.[Solomon *et al.* 2002]

We chose to investigate the strategy of pre-treatment of stimulator cells within a PBMC pool with stimulatory OKT3 and/or stimulatory anti-CD28 on alloantigen presentation and subsequent selective allodepletion based on responder CD69 expression.

OKT3 was chosen as a stimulatory anti-CD3 antibody. By mimicking antigen, anti-CD3 antibody can produce T cell activation but also anergy and death. Activation of resting T cells in vivo by anti-CD3 antibodies results in cytokine release both from T cells by cross-linking of T cell receptors and also from Fcγ receptor bearing cells (monocytes). Although anti-CD3 antibodies have been engineered with low Fcγ receptor binding affinities to minimise cytokine-induced toxicity when used as immunosuppressive treatment after solid organ transplantation, OKT3 has high Fcγ receptor binding affinity and was selected for use as any Fcγ receptor mediated cytokine release from non-T cells within the OKT3 treated PBMC pool might additionally increase the efficiency of alloantigen presentation. [Carpenter *et al.* 2000]

Immobilised OKT3 has been shown to be more effective than soluble OKT3 in stimulating mitogenesis and direct cytokine release from T cells. [van Lier *et al.* 1989]

The CD28 molecule is present on 95% of resting $CD4^+$ cells and 50% of resting $CD8^+$ cells.[June *et al.* 1990] Activation of T cells via antibody mediated ligation of CD28 has provided evidence for a CD28 signalling pathway which involves stabilization of cytokine mRNA levels and is separate from that used by the TCR-CD3 complex.[Siefken *et al.* 1997] Thus antibody mediated CD28 ligation of T cells may increase autocrine production of cytokines which might increase their expression of molecules important in presentation of alloantigens via an additional and separate pathway to CD3-mediated activation via OKT3. Treatment of T cells with stimulatory anti-CD28 antibody alone has been shown to cause activation and IL-2 secretion.

Proliferative responses and T cell survival after anti-CD3 antibody stimulation may be increased by additional co-stimulation with anti-CD28. [Boise *et al.* 1995;Meijer *et al.* 2001;Siefken *et al* 1997]

The initial hypothesis tested was that immobilised OKT3 +/-stimulatory anti-CD28 antibody stimulation might lead to activation of T cells and upregulation of expression on T cells of molecules involved in antigen presentation (Figure

3.1(A) and 3.1(B)), and an increase in the capacity of such T cells to present alloantigens in a one-way MLR.

T cells can stimulate apoptotic cell death via interaction between CD178 (FAS ligand) and CD95 (FAS) on target cells Expression of CD178 on T cells within a PBMC pool was measured, as this could provide an increased FAS-mediated signal to target cells in the one-way MLR and result in increased selective cell death of alloresponder cells. This pathway has been exploited (via the administration of exogenous anti-CD95 to responder cells) and has been found to effectively deplete alloreactive cells in an MHC-mismatched murine model and in human in vitro pairs.[Hartwig *et al* 2002;Nonn *et al.* 2003a]

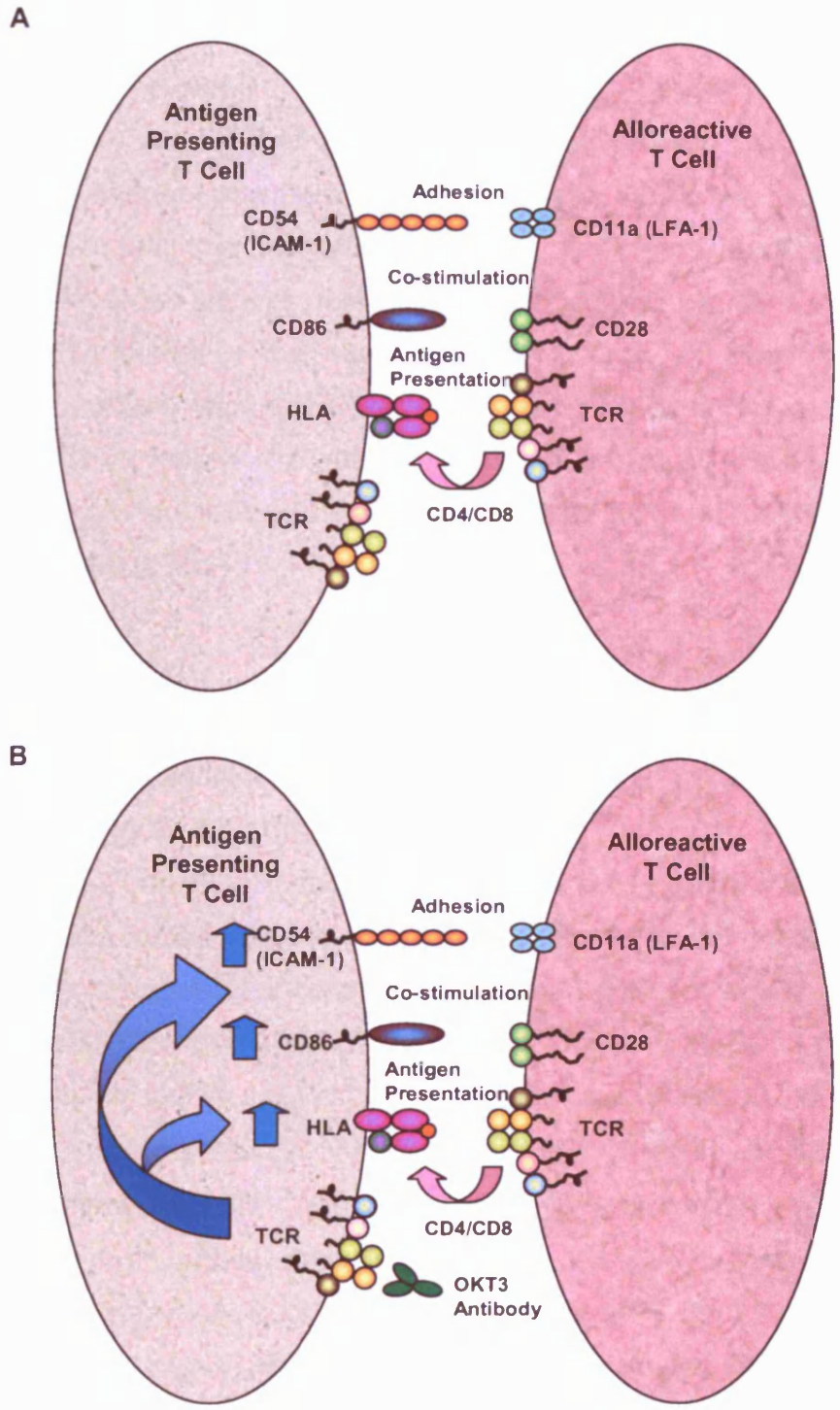

Figure 3.1 OKT3 pre-treatment of T cells and T-T antigen presentation.

(A) Molecules involved in presentation of alloantigens to alloreactive T Cells

(B) Possible mechanism of OKT3 in potentiating the capacity of T cells for presenting alloantigens.

3.2 Aims of Experiments Described in this Chapter

1. To examine the effect of OKT3 pre-treatment of T cells within the PBMC stimulator cell pool on their expression of various molecules important for the presentation of alloantigens;
2. To examine whether responder cell hyporesponsiveness and anergy was a frequent occurrence in the HLA-mismatched and HLA-matched setting;
3. To examine the effect of OKT3 pre-treatment of stimulators on the temporal kinetics of responder cell CD69 expression;
4. To compare the allostimulatory capacity of PBMCs in the cytokine modified MLR and OKT3 pre-treated PBMCs in HLA-matched pairs;
5. To compare the efficacy of CD69 based selective depletion in HLA-matched setting following the cytokine modified MLR and the OKT3 pre-treated MLR.

3.3 Methods

Human monoclonal mitogenic OKT3 was obtained from culture supernatant and quantified by ELISA (Chapter 2.8).

OKT3 was bound on to the surface of individual wells of multiple 24 well plates. After initial experiments to optimise the amount of OKT3 added to each well to result in stimulation of cells a standardised protocol was established in which 1ml of OKT3 supernatant (500ng) plus 2-4mg of anti-CD28 (stimulatory azide-free murine IgG2a anti-human CD28, Diaclone) was added to each well and plates incubated for 24 hours at 4°C (or 4 hours at 37°C). The OKT3 supernatant was then removed and each well washed with 1 ml of HBSS. OKT3-layered plates were kept at 4°C wrapped in foil and used within 7 days.[Duarte *et al.* 2002] 1 ml of fresh PBMCs from healthy donors at 10^6 cells/ml in complete medium was added to each well. The following wells were set up in triplicate:

Well	OKT3	Anti-CD28
1	None	None
2	500ng/well	None
3	None	2μg/well
4	None	4μg/well
5	500ng/well	2μg/well
6	500ng/well	4μg/well

Table 3.1 OKT3 +/- anti-CD28 stimulation of PBMCs.

After 24 and 48 hour culture periods, cells were incubated with the following panels of monoclonal antibodies and analysed by flow cytometry:

Panel/Fluorochrome	FITC	PE	PerCP
1	CD11a	CD54	CD3
2	HLA Class I	CD69	CD3
3	CD178	CD69	CD3
4	CD80*	CD86	CD3
5	HLA DR	CD54	CD3

*some subjects only

Table 3.2 Antibody panels used to measure expression of molecules important in presentation of alloantigens on OKT3-stimulated T cells.

PBMCs from 5-8 healthy donors were used. Antibodies for flow cytometric analysis other than CD178 (Bender Med Systems) and HLA Class I (Serotec) were supplied by BD. The staining procedure was as described in Chapter 2.2.1. CD178 was used at 1μL/ 2 x 10^5 cells and incubated on ice for 30 minutes prior to the addition of 500μL of HBSS, centrifugation at 200g for 10 minutes and resuspension in 300μL HBSS. The relative proportions of cell subsets within PBMCs were subsequently assessed at 0, 24 and 48 hours after

pre-treatment with OKT3 alone utilising the following cell panels (all BD antibodies)

Panel/Fluorochrome	FITC	PE	PerCP	APC
1	CD56	CD69	CD8	CD3
2	CD19	CD14	-	CD3

Table 3.3 Antibodies used to phenotype cells within PBMC pool before/after OKT3 stimulation.

The OKT3 stimulated proliferation of PBMCs (and the effect of γ-irradiation upon this) was assessed to ensure that OKT3 pre-treated stimulator cells did not proliferate after irradiation and were therefore suitable for use in the MLR. 10^5 fresh PBMCs/well (untreated or pre-treated with OKT3 for 48 hours) were incubated under standard conditions for 120 hours in 96 well plates and tritiated thymidine was added (1μCi/10^5 cells) at 108 hours. Proliferation was measured at 120 hours (Chapter 2.3.2). This was repeated with cells exposed to 30Gy γ-irradiation prior to incubation. To confirm that OKT3 pre-treated stimulator cells underwent sufficient apoptotic shrinkage after 30Gy of γ-irradiation to enable reliable differentiation from live responder cells in a FSC vs SSC plot, stimulator cells (either untreated or pre-treated with OKT3) were labelled with PKH-26 dye (see Chapter 2.9) prior to γ-irradiation and HLA-mismatched MLRs were set up. At 72 hours cells were labelled using the standard protocol with CD3-FITC, CD4-PerCP and CD69-APC. No PE fluorochrome was used to avoid interference with the PKH-26 dye, which fluoresces brightly in FL2.
The effect of OKT3 pre-treatment of stimulator cells in 16 HLA-mismatched (Table 3.4) and 16 HLA-matched (Table 3.5) stimulator-responder pairs was assessed. Responder cell CD69 expression and proliferation was measured and compared to that seen in the standard MLR and cytokine-modified MLR allostimulation strategies. Finally the efficiency of selective depletion of $CD69^+$ responders using the Miltenyi anti-FITC microbead system was compared after pre-treatment of stimulators with OKT3 and after the cytokine-modified MLR strategy in 14 of the HLA-matched stimulator-responder pairs using the schema set out in Figure 3.2

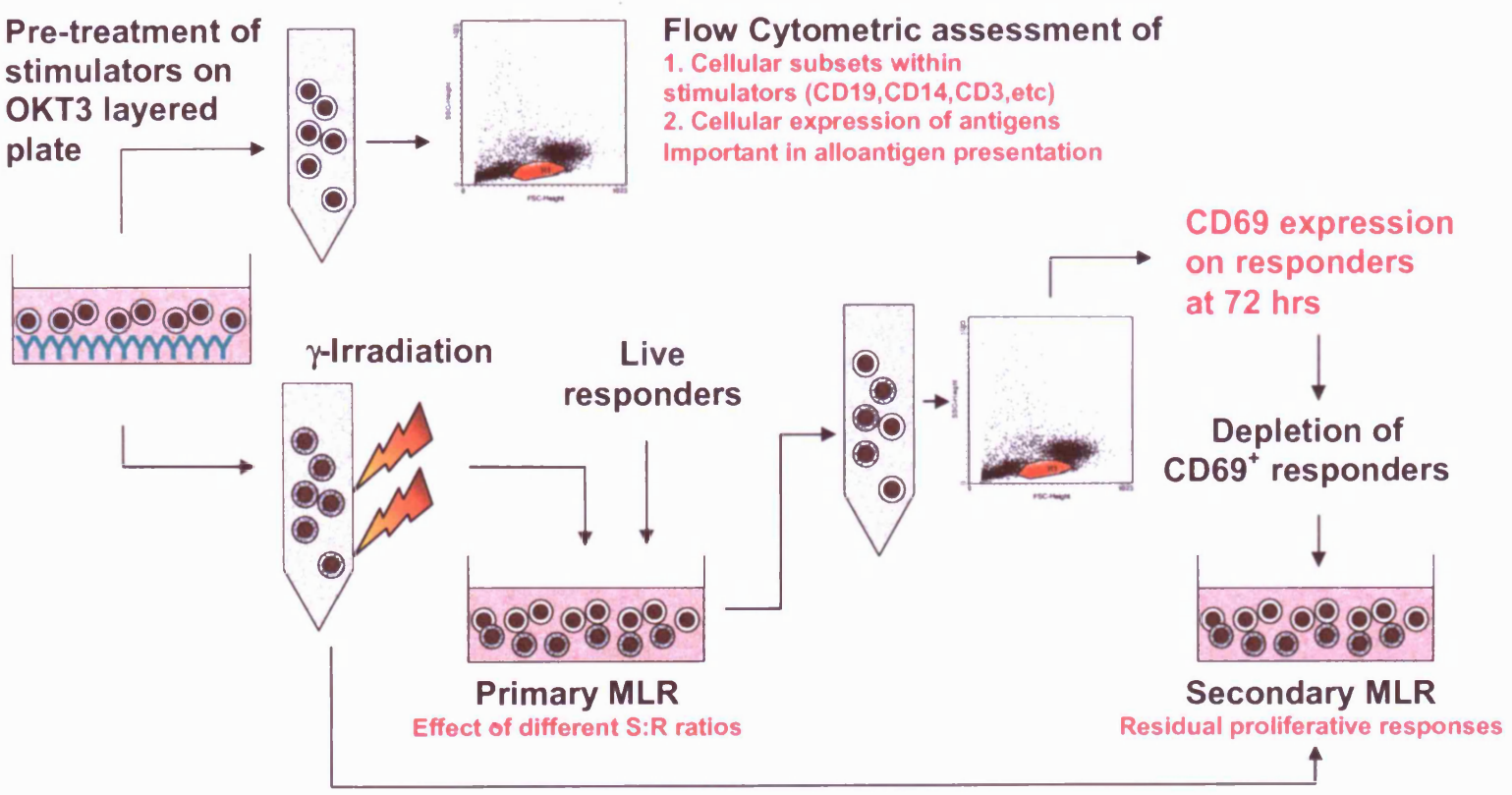

Figure 3.2 Experiments to investigate the effect of OKT3 on PBMC stimulators in the MLR.

Pair	Stimulator HLA type	Responder HLA type
1	A*0201,*24; B*5101,*4405; C*0202,*1502; DRB1*04, *11	A*0201, *01;B*0801,*40;C*01,*12 DRB1* 0403, *0404
2	A*0201,*3001;B*0702,*4402; C*07,*05; DRB1* 1401,*0810	A*0201,*302; B*52,*40 C*1502,*1202;DRB1*1502,*16
3	A*0201,*29;B*08,*15;C*07,*03; DRB1*0301,*13	A*0201,*0101;B*1402,*4101; C*17,*08; DRB1*0302,*0701
4	A*0201; B*08,*04; C*03,*07; DRB1*0101,*0301	A*0201,*6801;B*51,*0702; C*1502,*07; DRB1* 1501/13021
5	A*0201; B*57,*40; C*02,*06; DRB1*1101,*1104	A*0201,*33;B*58,*3508C*0302,*04; DRB1*1601,*0301
6	A*0201,*33; B*58,*3508 C*03,*04; DRB1*1601,*0301	A*0201,*3001;B*0702,*4402; C*07,*05; DRB1* 1401,*0810
7	A*0201,*24; B*5101,*4405; C*0202,*1502; DRB1*04, *11	A*0101; B*0801; C*0701; DRB1* 0301
8	A*0201,*24; B*5101,*4405; C*0202,*1502; DRB1*04, *11	A*0201,*24; B*0702,*1401; C*07,*08; DRB1*0401,*0701
9	A*0201,*24; B*5101,*1502; C*0202,*1502; DRB1*04,*11	A*0201,*1101;B*44,*27;C*02,*05; DRB1*1501,*0401
10	A*0201,*0101;B*1402,*4101; C*17,*08; DRB1*0302,*0701	A*02,*0101; B*08,*04; C*03,*07; DRB1*0101,*0301
11	A*0201,*6801;B*51,*0702; C*15,*07; DRB1*1501/13021	A*02,*01; B*57,*40; C*02,*06; DRB1*1101,*1104
12	A*0201,*302; B*52,*40 C*1502,*1202;DRB1*1502,*16	A*0201,*29; B*08,*15; C*07,*03; DRB1*0301,*13
13	A*0201,*24; B*5101,*4405; C*0202,*1502; DRB1*04, *11	A*0201,*0301;B*35,*37;C*04,*06, DRB1*0404,*1303
14	A*0201,*24; B*5101,*4405; C*0202,*1502; DRB1*04, *11	A*0101;B*0702,*3701;C*06,*07; DRB1*0404,*0701
15	A*0201,*24; B*5101,*4405; C*0202,*1502; DRB1*04, *11	A*0201,*31; B*44,*35; C*05,*12; DRB1*1501,*1401
16	A*0201,*24; B*5101,*4405; C*0202,*1502; DRB1*04, *11	A*0201,*0101;B*08,*18;C*03,*07; DRB1* 0301

Table 3.4 HLA typing of HLA-mismatched Stimulator-Responder pairs.

Pair	Stimulator HLA type	Responder HLA type
1	A, B, C, DR-matched SD	A*201, *01;B*0801,*40;C*01,*12 DRB1* 0403, *0404
2	A, B, C, DR-matched SD	A*0201,*302; B*52,*40 C*1502,*1202;DRB1*1502,*16
3	A, B, C, DR-matched SD	A*0201,*0101;B*1402,*4101; C*17,*08; DRB1*0302,*0701
4	A, B, C, DR-matched SD	A*0201,*6801;B*51,*0702; C*1502,*07; DRB1* 1501/13021
5	A, B, C, DR-matched SD	A*0201,*33;B*58,*3508C*0302,*04; DRB1*1601,*0301
6	A, B, C, DR-matched SD	A*0201,*3001;B*0702,*4402; C*07,*05; DRB1* 1401,*0810
7	A, B, C, DR-matched UD	A*0101; B*0801; C*0701; DRB1* 0301
8	A, B, C, DR-matched UD	A*0201,*24;B*0702,*1401;C*07,*08; DRB1*0401,*0701
9	A, B, C, DR-matched UD	A*0201,*1101;B*44,*27;C*02,*05; DRB1*1501,*0401
10	A, B, C, DR-matched UD	A*02,*0101; B*08,*04; C*03,*07; DRB1*0101,*0301
11	A, B, C, DR-matched UD	A*02,*01; B*57,*40; C*02,*06; DRB1*1101,*1104
12	A, B, C, DR-matched UD	A*0201,*29; B*08,*15; C*07,*03; DRB1*0301,*13
13	Single DRB1* mismatched UD	A*0201,*0301;B*35,*37;C*04,*06, DRB1*0404,*1303
14	Single A locus mismatched UD	A*0101;B*0702,*3701;C*06,*07; DRB1*0404,*0701
15	A and C locus mismatched UD	A*0201,*31; B*44,*35; C*05,*12; DRB1*1501,*1401
16	Single C locus mismatched UD	A*0201,*0101;B*08,*18;C*03,*07; DRB1* 0301

Table 3.5 HLA typing of fully or partially HLA-matched Stimulator-Responder pairs. SD=Sibling Donor, UD=Unrelated Donor.

3.4 Results

In experiments to ascertain the optimal dose of immobilised OKT3 for stimulation of the T cell pool within unselected PBMCs, activation of T cells was measured by the upregulation of CD69 and HLA DR. Expression of both CD69 and HLA DR were maximally up regulated with 500ng of immobilised OKT3 and 10^6 cells/well (data not shown).

For antigens not constitutively expressed on $CD3^+$ cells (CD69, CD54, HLA DR, CD86, CD178,), antigen expression was quantified as the percentage of $CD3^+$ cells positive for the antigen (MCF) $>10^1$. For antigens constitutively expressed on $CD3^+$ cells (HLA Class I, CD11a) density of antigen expression was quantified as MCF. Changes in antigen expression on $CD3^+$ cells are presented as mean fold increase in percentage positive or MCF (above baseline levels seen in complete medium only wells).

3.4.1 Expression of Molecules involved in Antigen Presentation on T cells after Stimulation with OKT3+/-anti-CD28 Antibody

3.4.1.1 Expression of CD69

Only 1.2% +/-0.7% of baseline $CD3^+$ cells expressed CD69.

The percentage of $CD3^+$ cells expressing CD69 increased in all subjects (mean 30-fold increase) with OKT3 stimulation at 24 hours (p=0.007) and a 40-fold increase at 48 hours (p=0.002).In 2 of 6 subjects tested CD28 alone increased the percentage of $CD3^+$ cells expressing CD69 at both 24 and 48 hours at both CD28 doses but no increase was seen in the other individuals. The combination of OKT3 and CD28 did not result in an increase in the percentage of $CD3^+$ cells expressing CD69 above that seen with OKT3 alone.

3.4.1.2 Expression of CD11a

CD11a was expressed on all $CD3^+$ cells at rest. CD11a density on $CD3^+$ cells was not significantly altered by OKT3 after 24 hours but was significantly increased after 48 hours. (1.6-fold increase, p=0.04). CD28 alone had no effect

on CD11a expression on $CD3^+$ cells and no additional effect when used in conjunction with OKT3.

3.4.1.3 Expression of CD54

Only 5.3% +/-3.6% of baseline $CD3^+$ cells expressed CD54. The percentage of $CD3^+$ expressing CD54 increased after co-incubation with OKT3 at 24 hours (4.9 fold increase, p=0.03) and at 48 hours (8 fold increase, p=0.002); the increase at 48 hours was significantly larger than the increase at 24 hours (p=0.04). CD28 alone had no effect and no additional effect when used in conjunction with OKT3.

3.4.1.4 Expression of HLA Class I

HLA Class I was expressed constitutively on baseline $CD3^+$ cells. HLA Class I density on $CD3^+$ cells was not significantly increased by OKT3 alone at 24 hours, however the combination of OKT3 and CD28 (at the higher dose used) significantly increased HLA Class I MCF of $CD3^+$ cells at 24 hours (1.8-fold increase, p=0.02). HLA Class I MCF of $CD3^+$ cells was increased by OKT3 stimulation (3.9 fold increase, p=0.02) at 48 hours. CD28 alone had no effect and CD28 in conjunction with OKT3 conferred no additional increase above that seen with OKT3 alone after 48 hours.

3.4.1.5 Expression of HLA DR

9% +/-4% of baseline $CD3^+$ cells expressed HLA DR. The percentage of $CD3^+$ cells expressing HLA DR was significantly increased with OKT3 (2.2 fold increase, p=0.006) at 24 hours and at 48 hours (5.4 fold increase, p=0.007). CD28 alone had no significant effect and conferred no addition increase when used in conjunction with OKT3.

3.4.1.6 Expression of CD86

2.5% +/-1.3% of baseline $CD3^+$ cells expressed CD86. The percentage of $CD3^+$ cells expressing CD86 was significantly increased by OKT3 stimulation alone at

48 hours (3.8 fold increase, p=0.002). CD28 alone had no effect. The combination of OKT3 and CD28 (at either dose) significantly increased the percentage $CD3^+$ $CD86^+$ at 24 hours (3.5 and 3.4 fold increase p=0.02) but conferred no additional increase above that seen with OKT3 alone after 48 hours.

3.4.1.7 Expression of CD178 (FAS ligand)

2.4%+/-2.3% of baseline $CD3^+$ cells expressed CD178. The percentage of $CD3^+$ cells expressing CD178 was not significantly increased after OKT3 for 24 hours but was significantly increased (6.5 fold increase, p=0.01) after 48 hours. CD28 alone had no significant effect and conferred no addition increase when used in conjunction with OKT3. The positive results are summarised in Figure 3.3

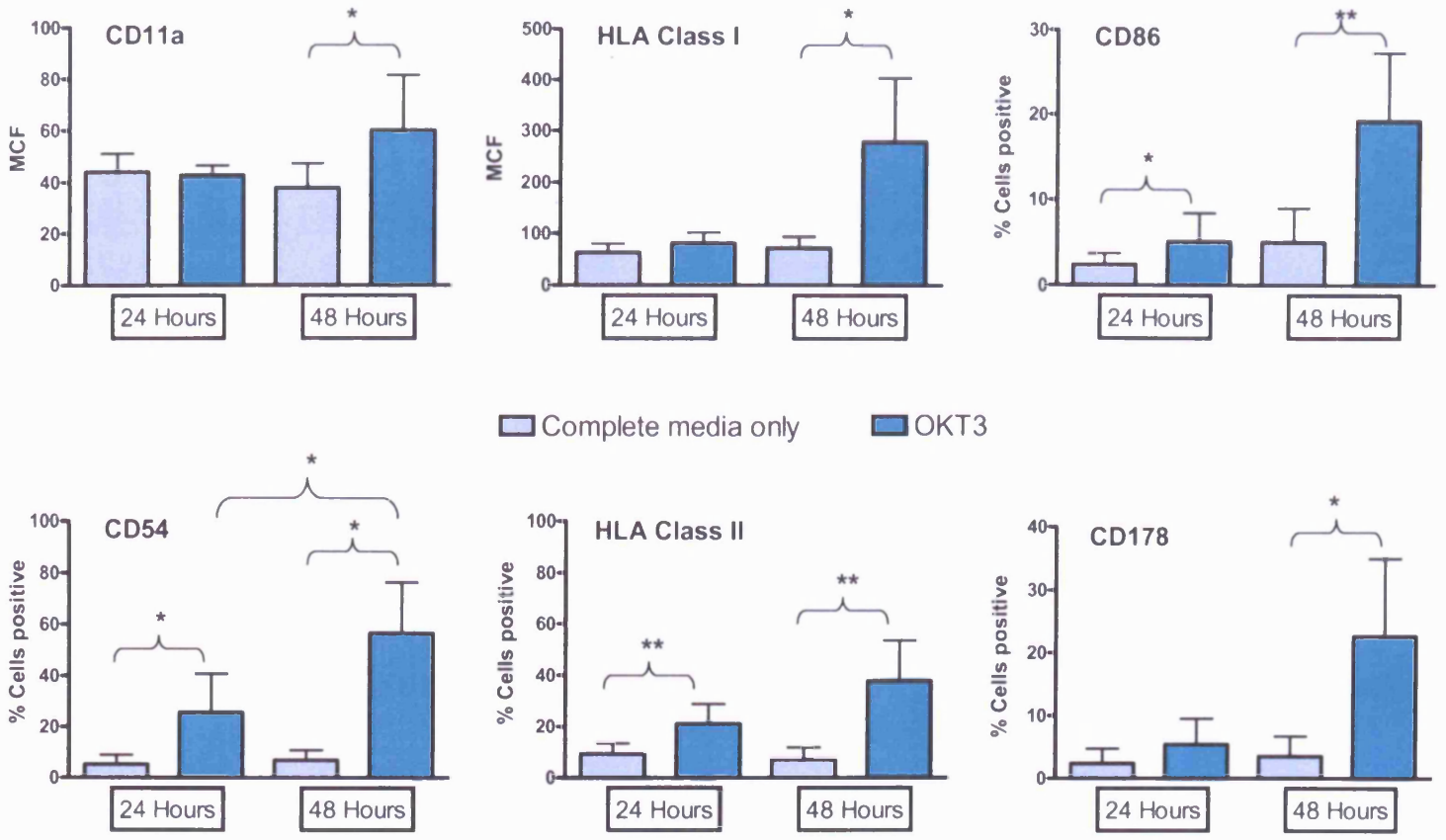

Figure 3.3 Expression of molecules important in antigen presentation on T cells after OKT3 pre-treatment for 24 and 48 hours. Error bars represent standard deviation. *=$p<0.05$,**=$p<0.01$ in unpaired two-tailed Student's t-tests.

3.4.1.8 Interpretation of Results

Following analysis of the results of the effects of OKT3 and/or anti-CD28 antibody on the expression on $CD3^+$ cells of molecules important in antigen presentation, a protocol of PBMC stimulation with 500ng immobilised OKT3/10^6 cells for 48 hours without additional CD28 stimulation was adopted for further experiments.

3.4.2 OKT3 Stimulation and Cell Subset Distribution

Healthy donor PBMCs were phenotyped with the antibody panel in Table 3.3, fresh and following incubation for 48 hours in OKT3 coated wells or in media only under standard conditions. Vigorous efforts were made to dislodge adherent monocytes from the wells after incubation for 48 hours and this was confirmed by light microscopy. Following acquisition of a minimum of 20,000 events on a FACSCalibur flow cytometer, a region was drawn on a FSC vs SSC dot-plot around live PBMCs (including monocytes) and the relative proportions of $CD3^+$, $CD19^+$, $CD14^+$ and $CD3^-CD56^+$ cells were calculated. $CD3^+CD56^+$ NK cells constitute a very small fraction of $CD3^+$ cells and were not included in the analysis, which was intended to demonstrate major changes in the proportions of T and B cells and monocytes. The results are presented are the mean cell frequencies from three different healthy donors. Compared to values in fresh PBMCs, the proportion of $CD3^+$ cells doubled after 48 hours of OKT3 stimulation (73% vs. 34%), the proportion of $CD19^+$ cells reduced by 25%, the proportion of $CD3^-CD56^+$ NK cells halved (2.1% vs. 4.3%) and the proportion of $CD14^+$ monocytes was greatly reduced (36.2% vs. 4.1%). However similar changes in proportions of all cell subsets with OKT3 stimulation were also seen after 48 hours of incubation in complete media alone, suggesting that the increase in proportion of $CD3^+$ T cells seen over this period was related to a growth advantage in cell culture and not directly related to the OKT3 stimulation. The reduction of $CD14^+$ monocytes might be due in part to retention of adherent cells in wells despite vigorous efforts to dislodge adherent cells prior to phenotyping. It is of note that OKT3 stimulation led to persistence of some $CD14^+$ monocytes, which still made up 4.1% of cells in the viable stimulator cell population. These results are illustrated Figure 3.4.

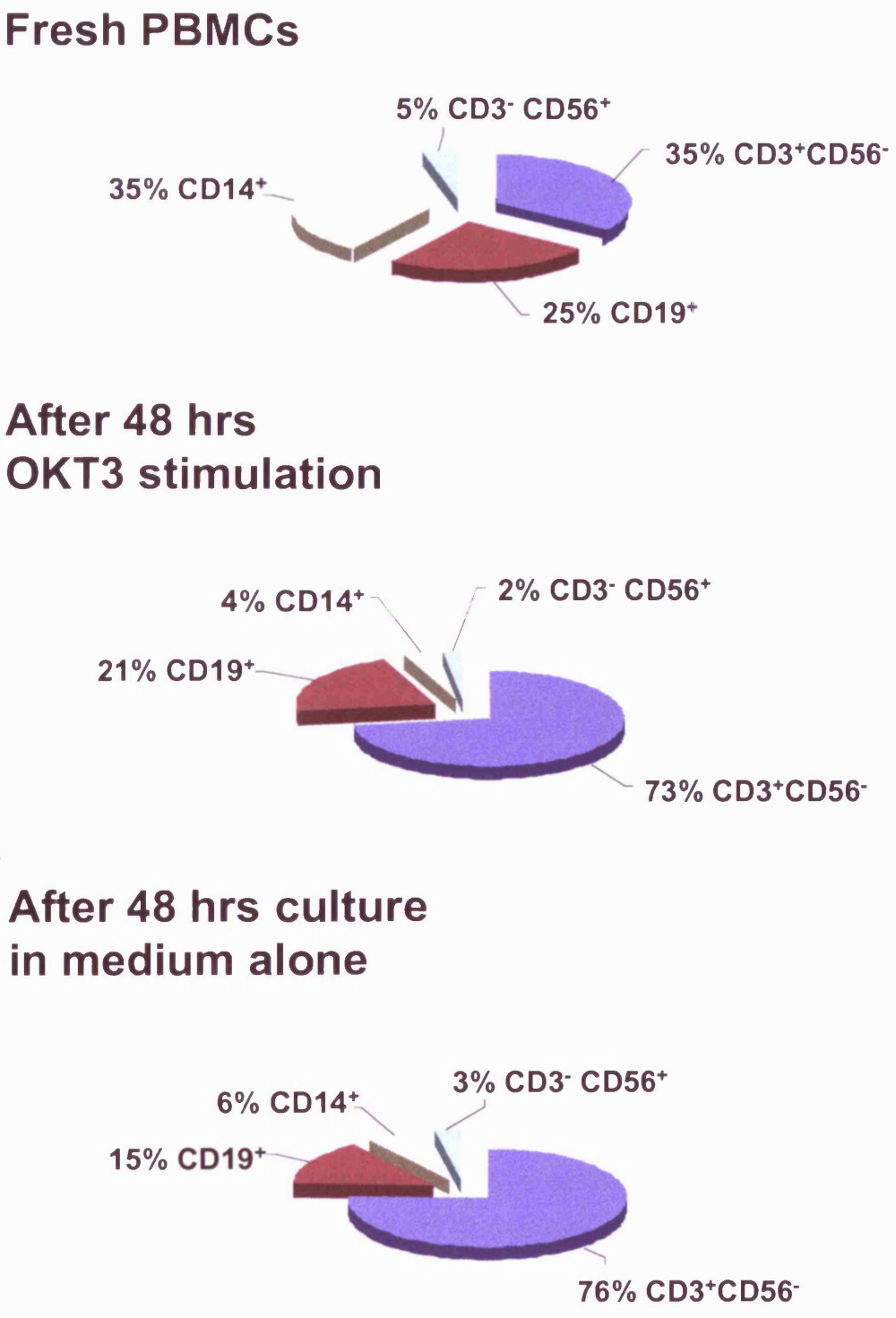

Figure 3.4 The effect of OKT3 stimulation on the relative proportions of cells within the PBMC pool.

3.4.3 Sensitivity to γ-Irradiation of OKT3 Pre-treated T cells

Before the effects of the OKT3 stimulator cell pre-treatment strategy on responder cells could be further investigated it was crucial to ascertain whether assumptions true for the standard MLR also held true for the OKT3 strategy. OKT3 pre-treatment is highly mitogenic and might render T cells more resistant to γ-irradiation and it was therefore important to determine the γ-irradiation dose required to abrogate proliferation of OKT3 pre-treated cells prior to use as stimulators in an MLR. Tritiated thymidine incorporation by untreated PBMCs from two healthy donors was measured at 72 hours before and after exposure to 30Gy of γ-irradiation and the experiment was repeated on PBMCs pre-treated for 48 hours with immobilised OKT3. 3-9 proliferation assays were performed per experiment.

	Proliferation, Tritiated Thymidine Uptake at 72 hours, cpm (mean+/-sd)			
Irradiation	None	+30Gy	None	+30Gy
OKT3 Pre-Treatment	No	No	Yes	Yes
Pair 1	*2264+/-441*	*574.3+/-261*	*16620+/-2345*	*325+/-129*
Pair 2	*1219+/-1224*	*340.6+/-138*	*53481+/-5036*	*1564+/-645*

Table 3.6 The effect of OKT3 stimulation on proliferation of unirradiated and irradiated PBMCs

Minimal proliferation was seen with untreated PBMCs. OKT3 pre-treatment of PBMCs stimulated proliferation, with tritiated thymidine uptake significantly higher than that seen with untreated PBMCs ($p<0.001$). 30Gy of irradiation significantly reduced proliferation of OKT3 pre-treated cells ($p<0.01$) to levels not significantly different from that seen in untreated PBMCs after 30Gy of irradiation. A dose of 30Gy of γ-irradiation was chosen to irradiate OKT3 pre-treated stimulators prior to their use in the MLR

3.4.4 Discrimination of Stimulator and Responder Cells

OKT3 pre-treatment of stimulator cells led to activation in a large proportion of cells (47%+/-14% of $CD3^+$ cells expressing CD69) and many of these cells enlarged as they underwent blastoid transformation (as determined by FSC and SSC signals by flow cytometry). OKT3 treated PBMC were seen to shift to the right of a FSC vs SSC dot plot on flow cytometric analysis. To confirm that irradiated OKT3 pre-treated stimulator cells underwent sufficient apoptosis by 72 hours to allow differentiation from live responders on a FSC vs SSC dot-plot, stimulator cells were labelled with PKH-26 dye prior to irradiation. The results after 72 hours of MLR co-culture with OKT3 pre-treated stimulators are shown in Figure 3.5. Figure 3.5 (A) shows a FSC vs SSC plot with apoptotic irradiated stimulator cells within region R1 and live responder cells within region R2. Figure 3.5 (B) is gated on the region R1 and shows 70% of cells within R1 were PKH^+ stimulator cells and 30% were apoptotic PKH^- responder cells. Figure 3.5 (C) is gated on R2 and shows that 98% of cells within this region were PKH^- and only 2% were PKH^+ stimulator cells. The low level of stimulator cells that contaminated the responder cell region is demonstrated in Figure 3.5 (E) which shows a FSC vs SSC dot plot back-gated on PKH^+ stimulator cells (pink) or PKH^- responder cells (lilac) from the dot plot in Figure 3.5 (D) after 72 hours of co-culture.

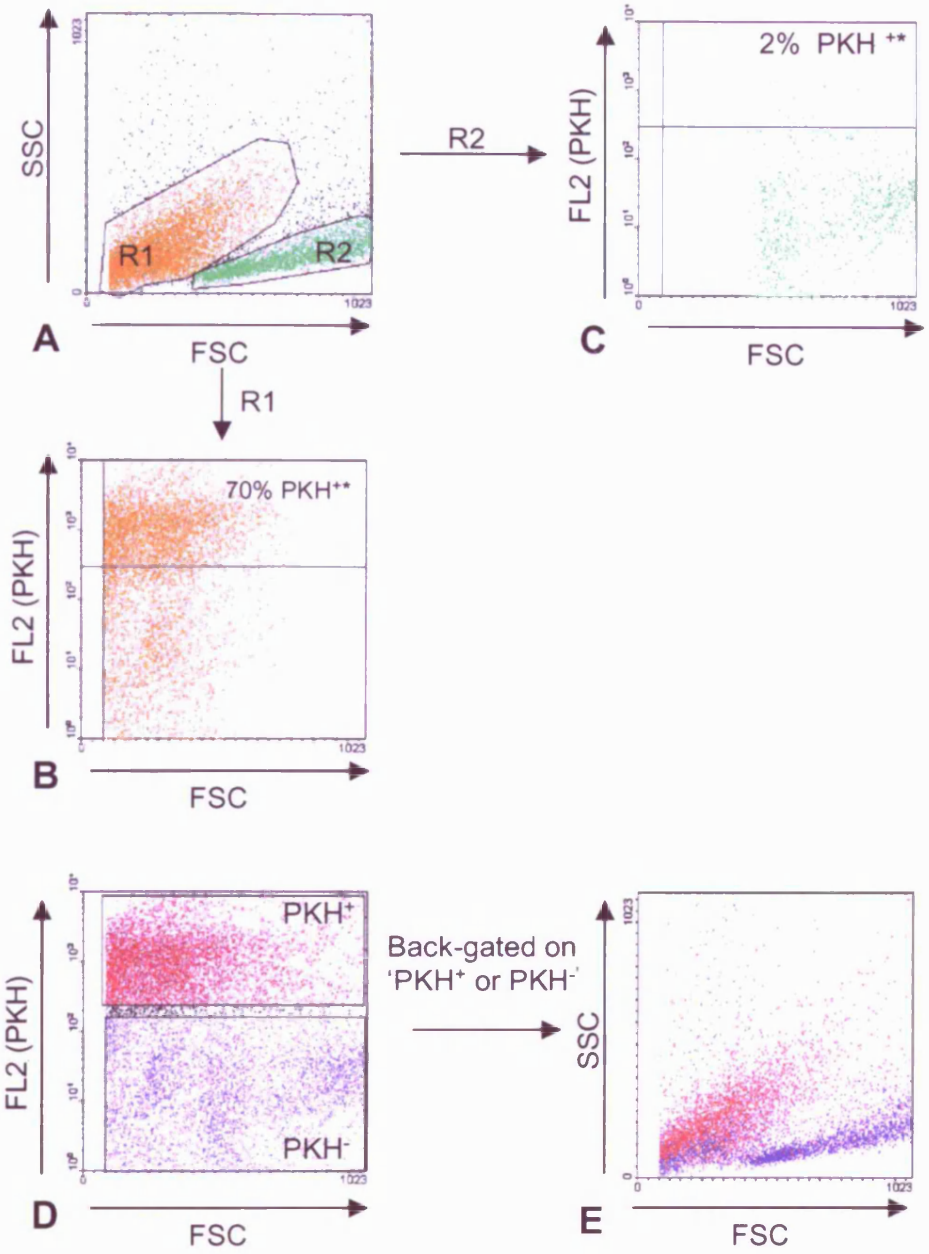

Figure 3.5 Discrimination of Stimulator and Responder Cells after OKT3 pre-treatment of stimulators. Stimulator cells were labelled with PKH-26 dye.

(A). FSC vs. SSC dot plot with stimulator (R1) and responder (R2) regions

(B). 70% of cells within R1 are PKH^+ stimulator cells

(C). 2% of cells within R2 are PKH^+ stimulator cells

(D). Regions of PKH^+ and PKH^- cells

(E). Back gated FSC vs. SSC dot plot demonstrating where PKH^+ and PKH^- cells lie

* above background level seen in unlabelled cells

3.4.5 OKT3 Pre-treated Stimulators in the MLR

The strategy of OKT3 pre-treatment of stimulator cells was assessed in the CD69 selective allodepletion protocol and compared with the use of cytokine-modified stimulators. Pre-treatment with 500ng immobilised OKT3/10^6 cells for 48 hours was chosen. Responder cell CD69 expression at 72 hours and proliferation (tritiated thymidine incorporation) at 120 hours was measured in 16 HLA-mismatched and 16 fully or partially HLA-matched stimulator-responder pairs.

OKT3 pre-treatment of stimulator cells led to significantly higher expression of CD69 on $CD3^+CD4^+$ and $CD3^+CD8^+$ responders in HLA-mismatched MLRs compared to untreated stimulators ($p<0.0001$ and 0.0003 respectively) or cytokine pre-treated stimulators ($p=0.0001$ and $p=0.002$ respectively). Cytokine pre-treatment led to a smaller, non-significant increase in CD69 expression on both cell subsets of HLA-mismatched responders when compared to unmodified stimulators.(Table 3.7)

Responder Cells	% CD69+ (above autologous control) (Mean +/-sd)		
	Standard MLR	Cytokine MLR	OKT3 MLR
$CD3^+$	*4.6%+/-3.7%*	*4.8%+/-3.7%*	*23%+/-14%*
$CD3^+CD4^+$	*4.4%+/-3.1%*	*5.2%+/-3.9%*	*27%+/-15%*
$CD3^+CD8^+$	*4.5%+/-3.4%*	*5.3%+/-5.3%*	*16%+/-10%*

Table 3.7 CD69 expression on HLA-mismatched responder cells (above autologous control) with untreated (standard), cytokine pre-treated and OKT3 pre-treated stimulator cells. Figures are mean +/- standard deviation.

Responder CD69 expression was greater following OKT3 pre-treatment of stimulator cells than with untreated stimulator cells in all pairs tested in $CD3^+CD4^+$ cells and in all but one pair in $CD3^+CD8^+$ cells. In contrast responder CD69 expression following cytokine pre-treatment of stimulator cells was unchanged in most pairs tested, with a marked increase (>33%) seen in both cell subsets in one pair (Pair 7), and a fall in expression in three pairs (Pair 2, Pair 3 and Pair 12). (Figure 3.6).

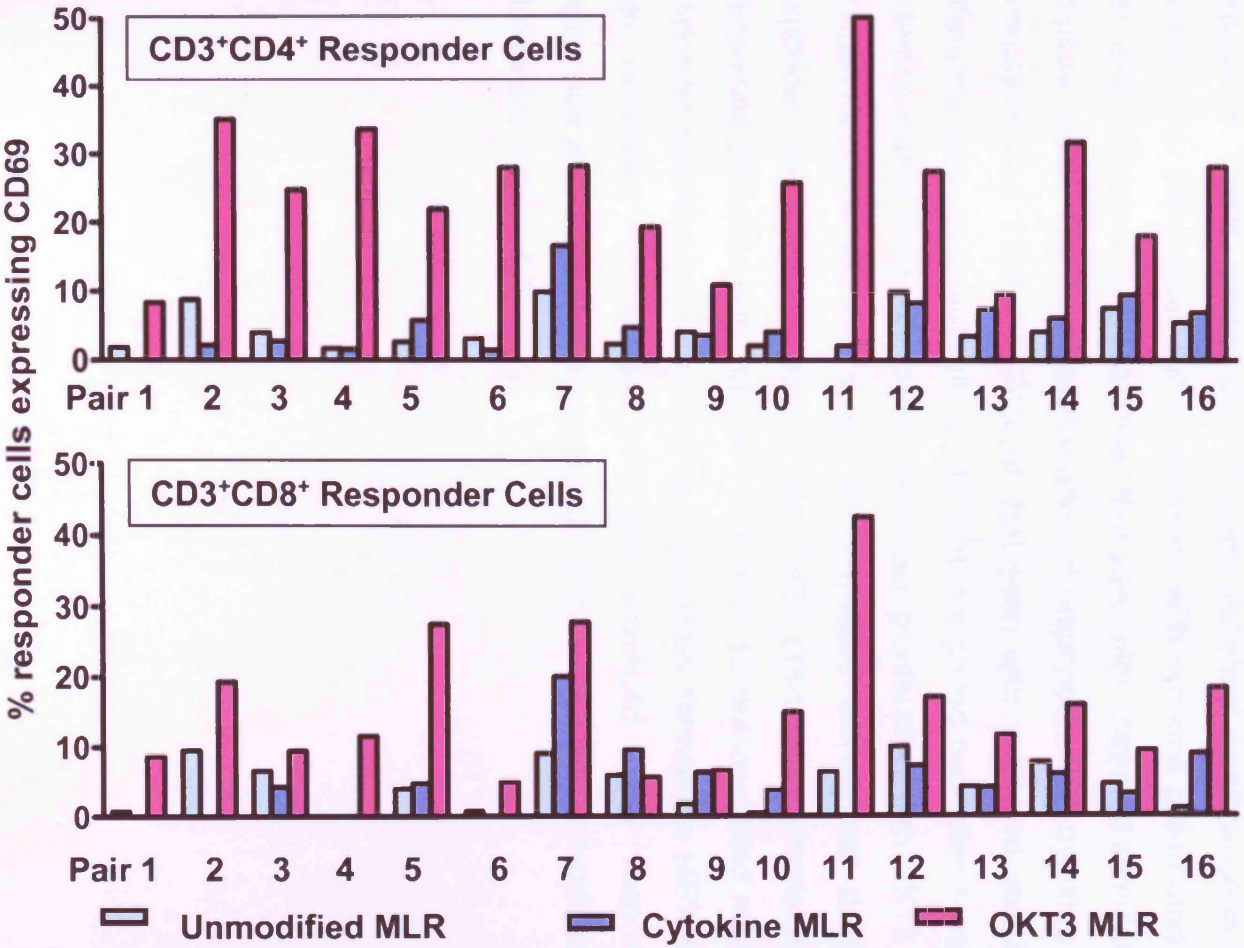

Figure 3.6 Responder cell CD69 expression in 16 HLA-mismatched MLRs after 72 hours stimulation with unmodified, cytokine pre-treated and OKT3 pre-treated stimulators.

3.4.5.1 Proliferation in HLA-Mismatched Pairs

Proliferation of HLA-mismatched responder cells with OKT3 pre-treated stimulators at 120 hrs was equivalent or greater than that seen with untreated stimulators in 14 of 16 pairs tested. The mean responder proliferation with OKT3 pre-treated stimulators was 157% +/- 54% of that seen with untreated stimulators. This difference did not reach statistical significance in a two-tailed paired t-test. Responder cell proliferation with cytokine pre-treated stimulators was also equivalent or greater than that seen with untreated stimulators in 14 of 16 pairs tested. The mean proliferation of responders with cytokine pre-treated stimulators was 111% +/- 24% of that seen with untreated stimulators. This difference was not statistically significant in a paired two-tailed t-test. Only Pair 5 demonstrated markedly lower responder proliferation with OKT3 pre-treated stimulators (32% of that seen with untreated stimulators) despite greater responder cell CD69 expression with OKT3 pre-treated stimulators than with unmodified stimulators in this pair. Only Pair 12 demonstrated markedly lower responder proliferation with cytokine pre-treated stimulators (46% of that seen with untreated stimulators); this pair exhibited lower responder CD69 expression with cytokine pre-treated stimulators than with unmodified stimulators (Figure 3.7).

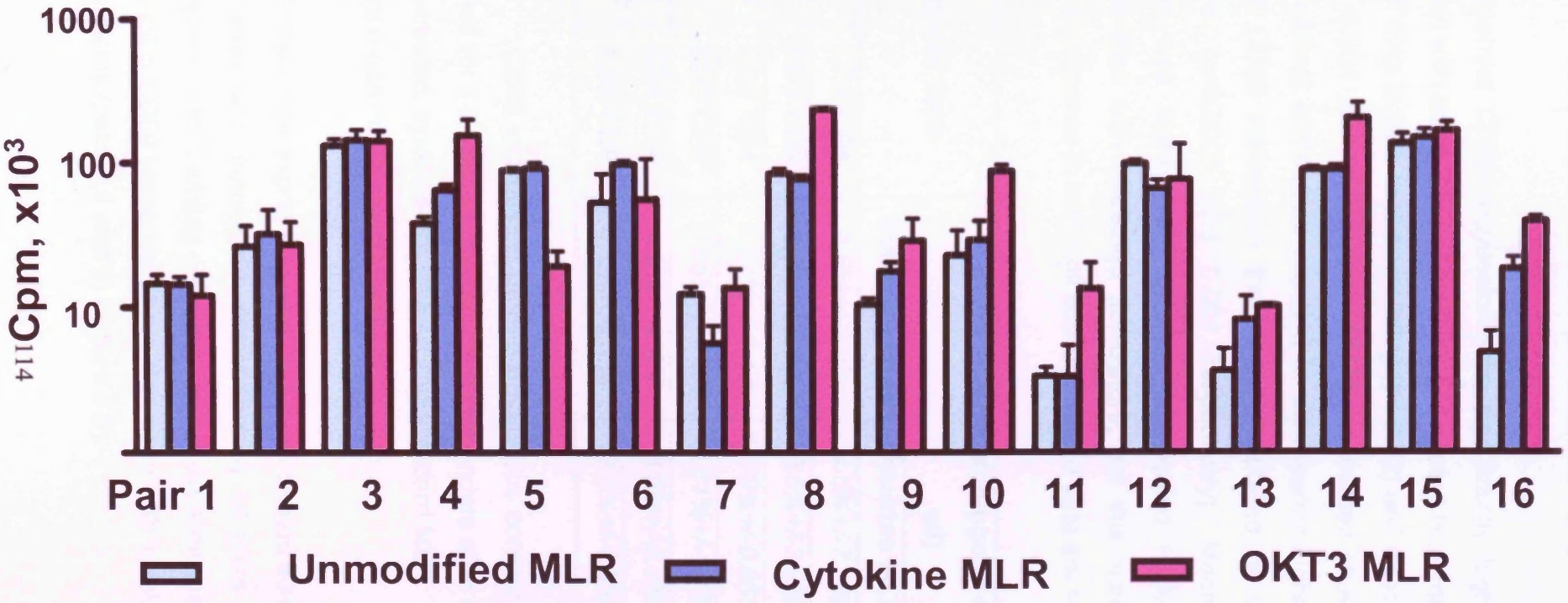

Figure 3.7 Responder cell proliferation in 16 HLA-mismatched MLRs after 120 hours of stimulation with unmodified, cytokine pre-treated and OKT3 pre-treated stimulators. Error bars represent standard deviation of triplicate proliferation assays.

3.4.5.2 CD69 Expression in HLA-Matched Pairs

Mean responder CD69 expression was significantly higher with OKT3 pre-treated than with unmodified or cytokine pre-treated HLA-matched stimulators in $CD3^+CD4^+$ cells in both matched sibling (p= 0.02) and matched unrelated donor pairs (p= 0.004 and 0.002). Only OKT3 pre-treated HLA-matched unrelated (and not sibling) stimulators resulted in significantly higher mean $CD3^+CD8^+$ responder CD69 expression than with unmodified or cytokine pre-treated stimulators, (p=0.0004 and 0.001 respectively). Mean responder CD69 expression was higher with cytokine pre-treated HLA-matched unrelated stimulators than with untreated stimulators, but this increase did not reach statistical significance in both cell subsets. These data are shown in Table 3.8.

Responder Cells		% $CD69^+$ (above autologous control) (mean +/- sd)		
		Standard MLR	**Cytokine MLR**	**OKT3 MLR**
All Matched Pairs	**$CD3^+CD4^+$**	*1.5%+/-2.5%*	*4.6%+/-7.1%*	*25.8%+/-12.4%*
	$CD3^+CD8^+$	*1.4%+/-2.4%*	*2.5%+/-3.9%*	*8.8%+/-6.4%*
Matched Sibling Pairs	**$CD3^+CD4^+$**	*0.97%+/-1.4%*	*0.79%+/-0.95%*	*23.7%+/-10%*
	$CD3^+CD8^+$	*0.95%+/-1.8%*	*0.61%+/-1.1%*	*10.9%+/-6.4%*
Matched Unrelated Pairs	**$CD3^+CD4^+$**	*1.9%+/-2.9%*	*6.9%+/-8.2%*	*22.1%+/-11%*
	$CD3^+CD8^+$	*1.6%+/-2.7%*	*3.5%+/-4.5%*	*12.6%+/-6.1%*

Table 3.8 CD69 expression (above autologous control) in 16 HLA A, B, C DR-matched (or 1-2 antigen mismatched) responders after allostimulation in the OKT3 pre-treated, cytokine pre-treated and standard MLR.
Figures are mean +/- standard deviation.

The CD69 responder expression with OKT3 pre-treated stimulators was greater than that seen with autologous stimulators in all pairs tested in both cell subsets, whereas in 3 sibling pairs the use of cytokine pre-treated stimulators failed to elicit a CD69 response above that seen with autologous stimulators in both cell subsets (pairs 3,4 and 5) (Figure 3.8).

No significant difference was seen in mean CD69 responder expression with OKT3 pre-treated stimulator cells *between* sibling pairs (n=6) or unrelated pairs (n=10) in both cell subsets. However the cytokine pre-treatment of stimulators resulted in significantly greater mean CD69 responder expression in unrelated pairs than sibling pairs in $CD3^{+}CD4^{+}$ cells (p= 0.04). Subdividing the matched unrelated donor group into full A, B, C and DR matches and those with 1 (or more) antigen mismatch did not yield any significant differences in CD69 expression on responder cells between these groups for either strategy.

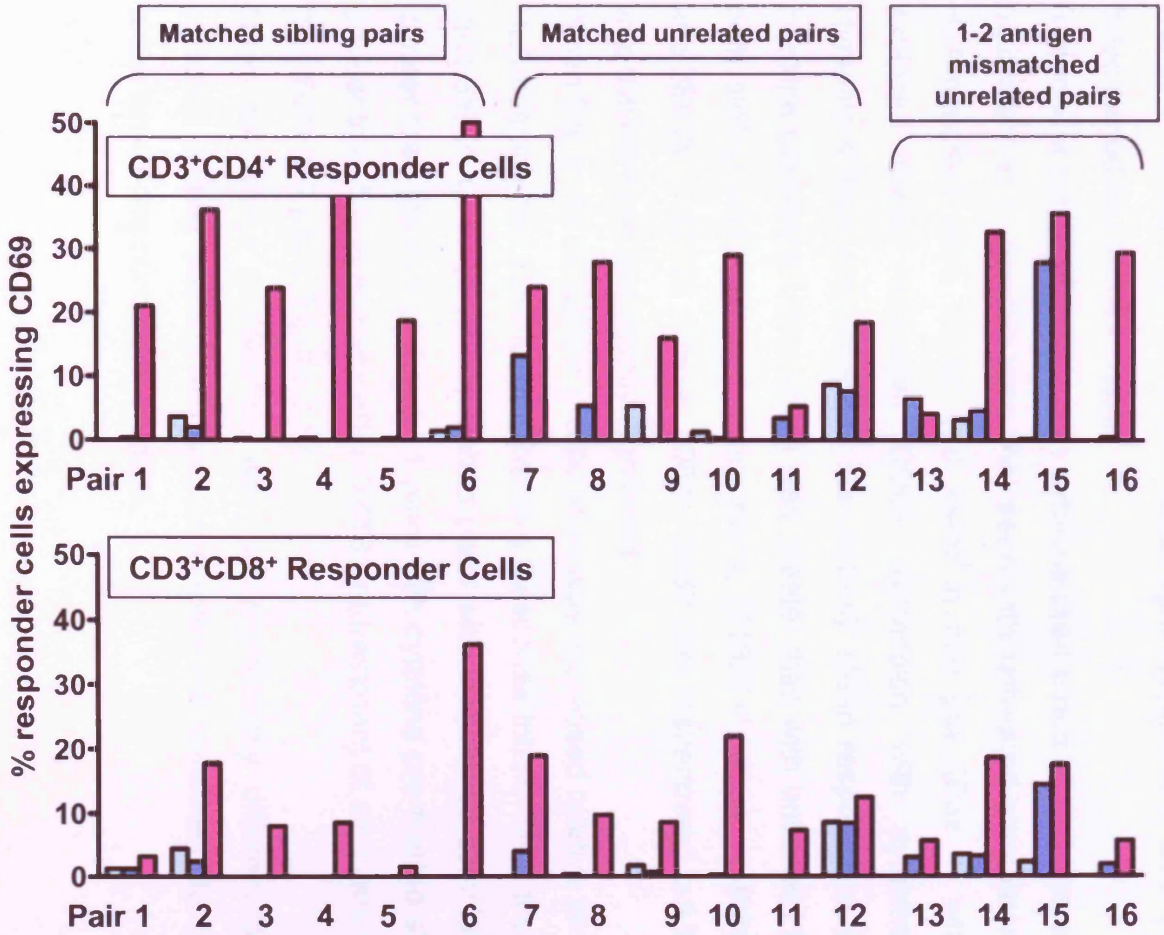

Figure 3.8 Responder CD69 expression in 16 HLA-matched (6 sibling and 10 unrelated) MLRs with unmodified, cytokine pre-treated and OKT3 pre-treated stimulators.

3.4.5.3 Proliferation in HLA-Matched Pairs

Responder cell proliferation with OKT3 pre-treated stimulators at 120 hrs was equivalent to or greater than that seen with untreated stimulators in all HLA-matched pairs. Mean responder proliferation with OKT3 pre-treated stimulators was significantly greater than with untreated stimulators for both sibling and unrelated donor matched pairs, (59631 +/-48244 vs.4084+/-5852 cpm and 53439 +/- 54359 cpm vs 4084+/-5852 cpm respectively, both p=0.02 in two-tailed paired Student's t-tests).

Responder cell proliferation with cytokine pre-treated stimulators at 120 hrs was equivalent to or greater than that seen with untreated stimulators in 14 of 15 HLA-matched pairs tested and lower in one pair (Pair 1, which had also exhibited lower responder CD69 expression with cytokine pre-treated stimulators than with untreated stimulators). Mean responder proliferation with cytokine pre-treated stimulators was greater than with untreated stimulators for both sibling and unrelated donor pairs, (21896+/-14304 vs.4084+/-5852 cpm and 28803 +/- 42963 cpm vs. 4084+/-5852 cpm respectively) but in neither case was the increase statistically significant.

When first party responder proliferation was expressed relative to that seen with HLA-mismatched stimulators (Relative Response Index, RRI), the RRI was less than 5% in 11 of 15 HLA-matched pairs with unmodified stimulators. RRI was greater than 5% in 9 of these 11 pairs with cytokine pre-treated stimulators and greater than 5% in all pairs after OKT3 pre-treatment of stimulators, and greater than 50% in 9 pairs (Figure 3.9).

Mean responder proliferation was not significantly different between HLA-matched sibling pairs (n=6) and HLA-matched unrelated donor pairs (n=9) under any of the conditions studied.

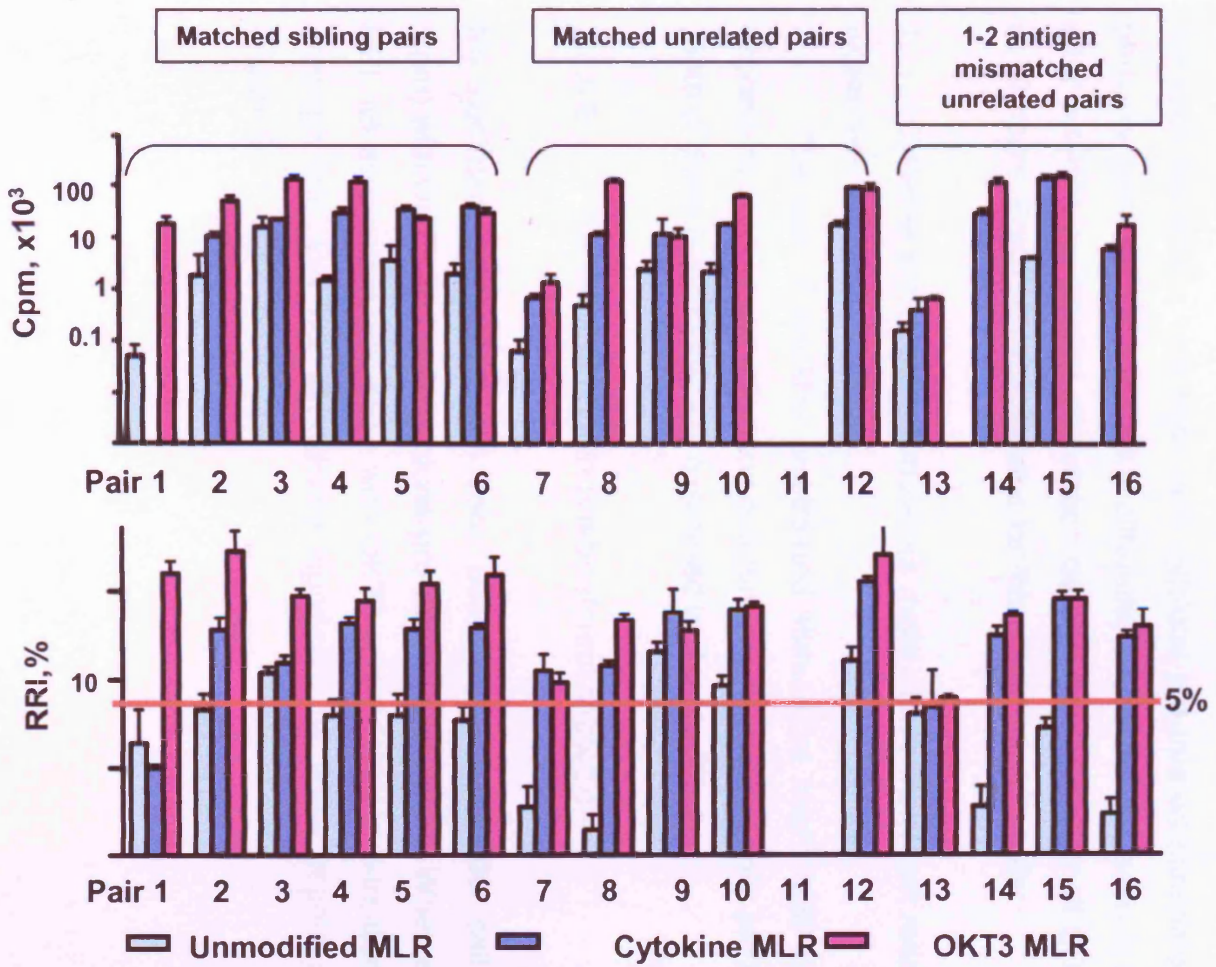

Figure 3.9 Responder proliferation at 120 hours in 16 HLA-matched pairs with unmodified, cytokine pre-treated and OKT3 pre-treated stimulators: cpm (top) and percentage RRI (bottom) Error bars represent standard deviation. Proliferation assays failed in pair 11.

3.4.5.4 Correlation of CD69 Expression and Proliferation

CD69 expression at 72 hours in HLA-matched $CD3^+CD4^+$ responders stimulated with OKT3 pre-treated cells correlated with the log of responder cell proliferation (assayed by tritiated thymidine uptake) at 120 hours. (Figure 3.10)
This is in keeping with published data that $CD4^+$ cell responses correlate most closely with proliferation in the MLR.[Nikaein *et al.* 1984]
No such correlation was seen with cytokine pre-treated stimulators. This was primarily because a substantial proliferative response was seen in several pairs after cytokine pre-treated stimulation despite only a very small CD69 response at 72 hours. Possible explanations for this observation include:

1. Cytokine pre-treated stimulators might cause maximum responder CD69 expression later than 72 hours.
2. The use of cytokine pre-treated stimulators might lead to preferential expression of alternate activation antigens on alloreactive cells (eg CD25)
(Both of these hypotheses are examined in Chapter 5).

3.4.5.5 Correlation of both pre-treatment strategies

No significant correlation was seen between the responder cell proliferation (cpm) with OKT3 and with cytokine-pre-treated stimulators. When expressed as RRI, an association was seen with OKT3 and cytokine pre-treated stimulators, although this was not statistically significant by linear regression analysis. (Figure 3.11).

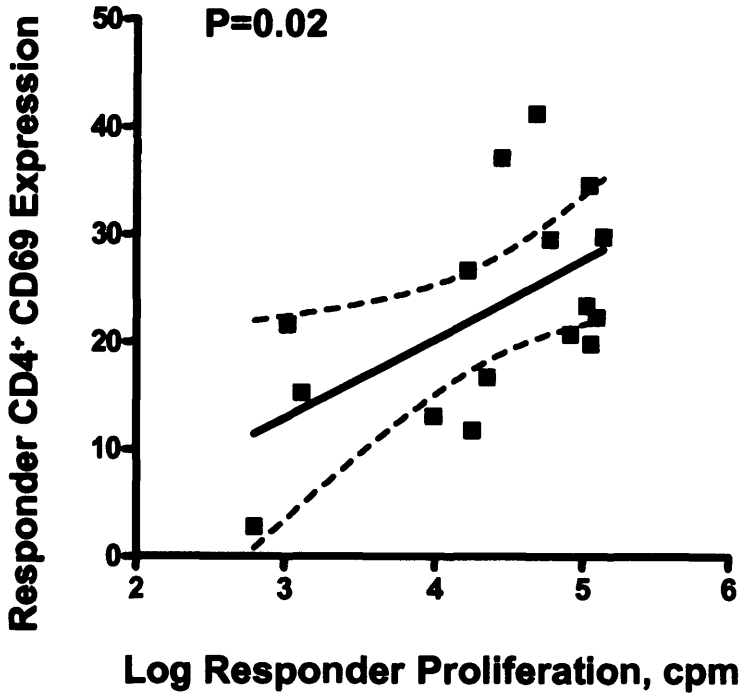

Figure 3.10 Correlation of $CD4^+$ responder CD69 expression (above autologous control) at 72 hours of co-culture and responder proliferation (log_{10} cpm) at 120 hours with OKT3 pre-treated stimulators.

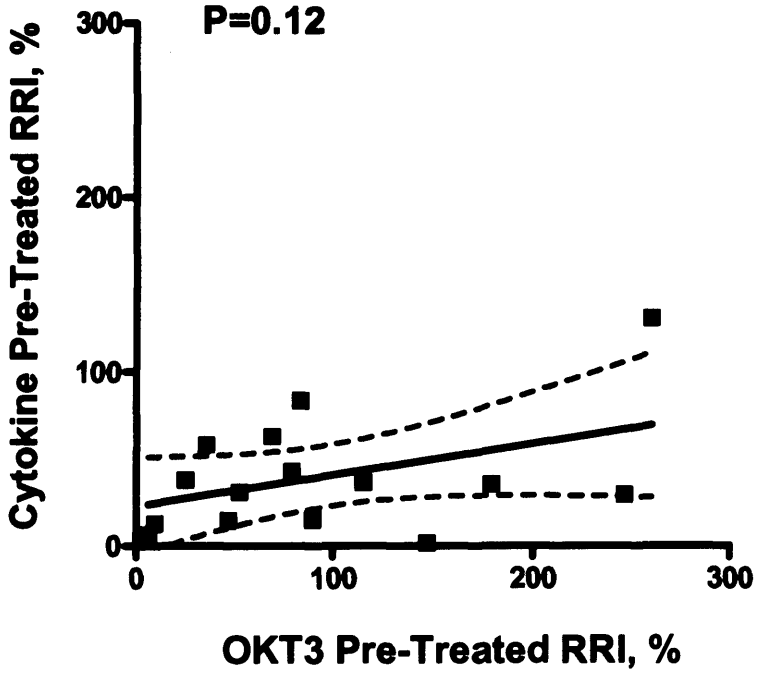

Figure 3.11 Correlation of OKT3 and cytokine MLR RRIs.

3.4.5.6 Reducing the Stimulator: Responder Ratio

The feasibility of reducing the Stimulator: Responder (S: R) ratio on responder CD69 expression and proliferation was investigated in 4 HLA-matched pairs (Pairs 1,8,15 and 16).

Reducing the S: R ratio to below 1:1 reduced both the responder CD69 expression on $CD3^+CD4^+$ and $CD3^+CD8^+$ cells and responder proliferation in all 4 pairs tested, although different patterns were seen in different pairs.

Pair 1 (an HLA-matched sibling pair) had a RRI<5% at all S: R ratios using cytokine pre-treated stimulators. However with OKT3 pre-treated stimulators the RRI was 30% at an S: R ratio of 1:5 and 100% at a S:R ratio of 1:1.

Pair 15 (a single A antigen mismatched unrelated pair) gave an RRI<5% at a S: R ratios of 1:10 and 1:5, and 80% at an S: R ratio of 1:1 using cytokine pre-treated stimulators, whereas the RRI was <5% at an S: R ratio of 1:10, 30% at an S: R of 1:5 and 90% at S: R ratio of 1:1 using OKT3 pre-treated stimulators.

Pair 16 (a single C locus mismatched unrelated pair) gave an RRI>5% at S: R ratios of 1:2 and 1:1 but not at lower ratios using cytokine pre-treated stimulators, whereas an RRI of >10% was seen at all S: R ratios tested with OKT3 pre-treated stimulators.

Finally pair 8 (a matched unrelated donor pair) gave an RRI >5% at an S: R of 1:1 (but not at lower S: R ratios), whereas an RRI of >10% at all S: R ratios tested was seen with OKT3 pre-treated stimulators.

Thus the cytokine modified MLR was only seen to give positive readouts (RRI>5%) in pairs 15,16 and 8 at higher S: R ratios (1:2 or 1:1). Although the significance of levels of proliferative responses (and RRI) in the OKT3 modified MLR remains to be determined, proliferative responses and RRIs >10% were seen at lower S: R ratios in all pairs tested and in some pairs at S: R ratios of 10:1. The OKT3 modified MLR might therefore prove useful in amplifying alloreactive responses in HLA-matched pairs when only small numbers of stimulator cells are available for use for clinical scale allostimulation and allodepletion of donor T cells (Figure 3.12).

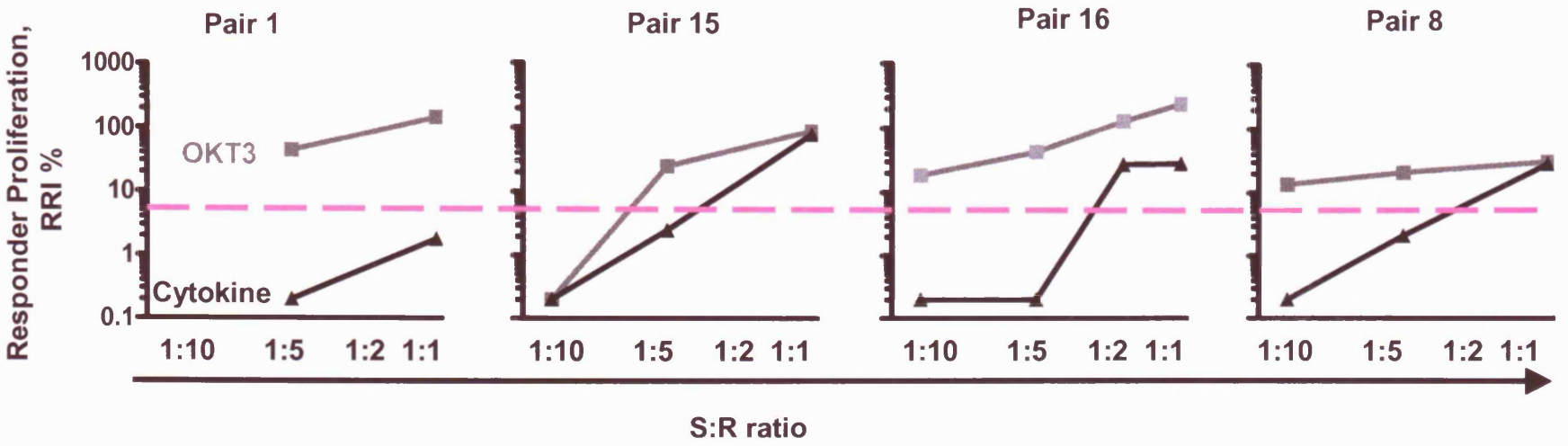

Figure 3.12 The effect of different S: R ratios on responder proliferation (expressed as RRI to HLA-mismatched third party stimulators) in 4 HLA-matched pairs. The pink dotted line represents a RRI of 5%, the level found to have a high negative predictive value for clinical GvHD in HLA-matched sibling pairs using the cytokine pre-treatment technique of allostimulation with an S: R ratio of 1:1.

3.4.6 Depletion of $CD69^+$ Cells in HLA-Matched Pairs

In 13 of the HLA-matched pairs, OKT3 and cytokine modified MLRs (Chapter 2.3.4 and 2.3.3) were depleted of $CD69^+$ alloreactive cells at 72 hours by MACS immunomagnetic sorting (Chapter 2.5.1) and responder proliferation to first and third party stimulators assessed pre- and post-depletion (Chapter 2.4.2).

Depletion efficiency in the OKT3 modified MLR was 93%+/-10% for $CD3^+CD4^+CD69^+$ cells and 92%+/-13% for $CD3^+CD8^+CD69^+$ cells. Depletion efficiency was similar in the cytokine modified MLR, (91%+/-10% for $CD3^+CD4^+CD69^+$ cells and 90%+/-11% for $CD3^+CD8^+CD69^+$ cells)(Figure 3.13).

Reduction in first party proliferative responses was excellent utilising the OKT3 modified MLR, with proliferative responses to first party stimulators using depleted responders reduced to 0.8%+/-2.1% of the pre-depletion value and RRI falling from a mean for all pairs of 69%+/-49% pre-depletion to 0.9%+/-2.4% post depletion. Third party proliferative responses were well maintained with a mean for all pairs tested of 87%+/-51% of the predepletion values. In one in pair tested the third party response was lost (post depletion value only 8.2% of the pre-depletion value) indicating failure of selective alloreactive depletion in this pair. If this pair is excluded the mean third party proliferative response post depletion was 93%+/-36% of that seen with unmanipulated cells.

Reduction in first party proliferative responses was good utilising the cytokine modified MLR, with proliferative responses to first party stimulators using depleted responders reduced to 2.1%+/-5.2% of the pre-depletion value and RRI falling from a mean for all pairs of 38%+/-35% pre-depletion to 0.7%+/-2% post-depletion. All pairs with a pre-depletion RRI >5% had a post-depletion RRI<5% using the cytokine allodepletion study. Third party proliferative responses were well maintained with a mean for all pairs tested of 99%+/-86% of the pre-depletion values (Figure 3.14).

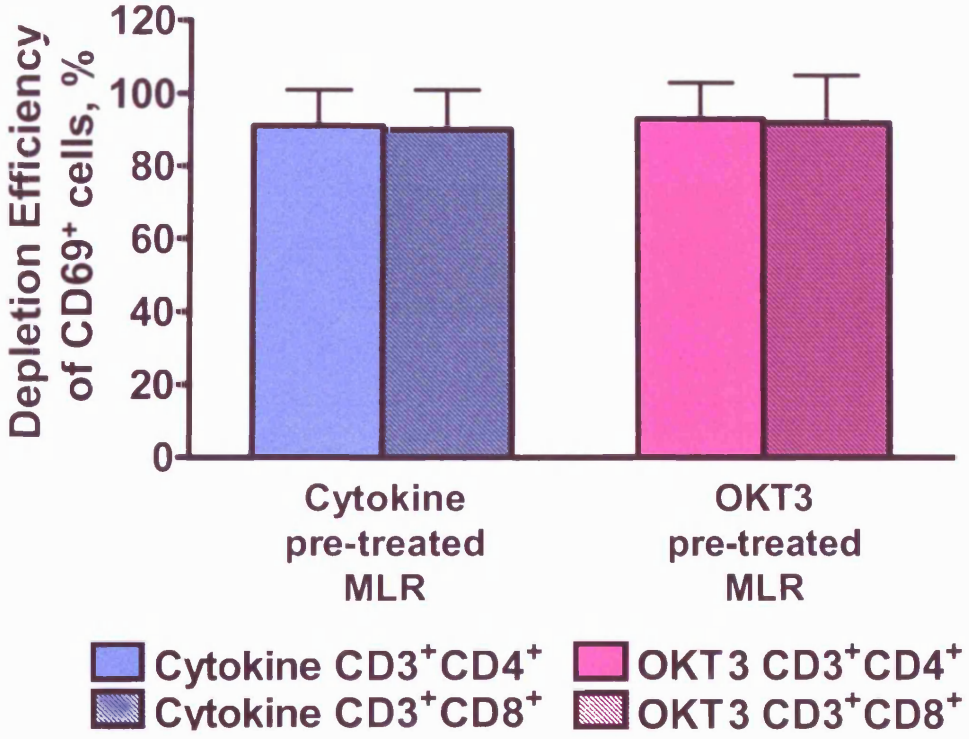

Figure 3.13 Efficiency of immunomagnetic depletion of $CD69^+$ cells after cytokine or OKT3 pre-treated allostimulation. Error bars represent standard deviation. Results are presented for 12 HLA-matched pairs.

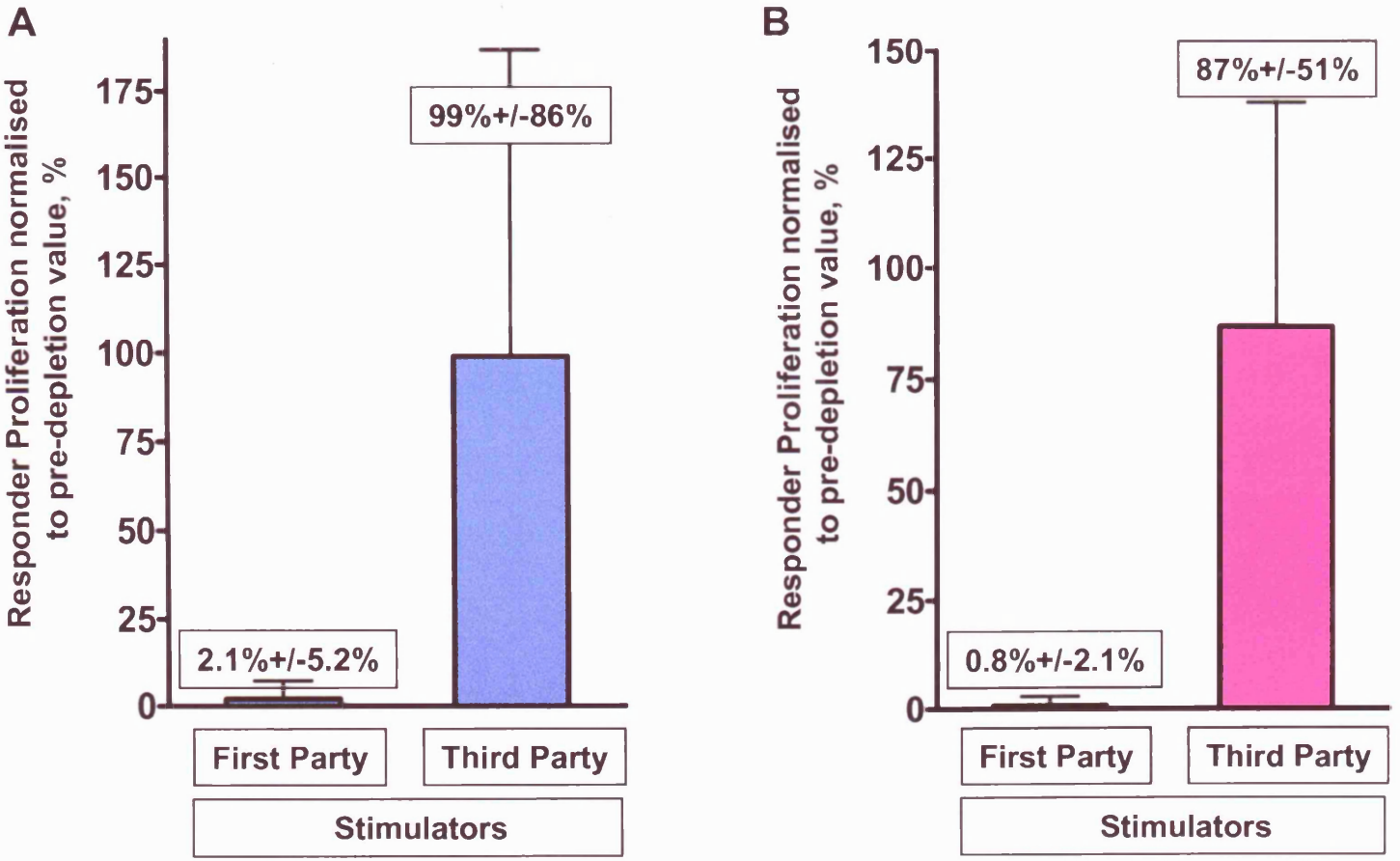

Figure 3.14 Responder cell proliferation pre- and post-depletion. Error bars represent standard deviation. Results are presented for 12 HLA-matched pairs. (A). Cytokine pre-treated stimulator cells (B). OKT3 pre-treated stimulator cells.

3.4.7 Correlation of MLR and GvHD in HLA-Matched Pairs

The results of proliferation in cytokine and OKT3-modified MLRs (expressed as RRI,%) are shown for the14 HLA-matched pairs tested from donor-recipients who went on to have AHSCTs are shown in Table 3.9, along with details of the stem cell source, transplant conditioning and acute GvHD.

Pair	Donor	Cytokine MLR RRI	OKT3 MLR RRI	Stem cell source	Conditio-ning	Acute GvHD
1	HLA-matched SD	1.8	147	TD* PBSC	Cy/TBI	1 (skin)
2	HLA-matched SD	33	179	NTD BM	Bu/Cy	4 (skin)
3	HLA-matched SD	15	90	NTD PBSC	Cy/TBI	4 (Liver)
4	HLA-matched SD	44	74	NTD PBSC	Flu/mel/ Campath	0
5	HLA-matched SD	36	115	NTD PBSC	Cy/TBI	1 (skin)
6	HLA-matched SD	39	24	NTD PBSC	Flu/Mel/ Campath	0
7	HLA-matched UD	11	9.5	NTD BM	Cy/TBI	0
8	HLA-matched UD	15	31	NTD PBSC	Flu/BEAM/ Campath	0
9	HLA-matched UD	62	34	NTD PBSC	Flu/Mel/ Campath	0
10	HLA-matched UD	59	69	NTD PBSC	Flu/Bu/ Campath	1 (skin)
13	HLA DRB1*04 m/m UD	4.6	6.25	NTD PBSC	Flu/BEAM/ Campath	2 (skin)
14	HLA A m/m UD	31	52	TD* PBSC	Cy/TBI	2 (skin)
15	HLA A and C m/m UD	83/80	89/89*	NTD BM	Flu/Treo/ Campath	2 (skin)
16	HLA C m/m UD	29	246	TD* PBSC	Cy/TBI	2 (skin)

Table 3.9 Details of HLA-matched pairs tested that underwent AHSCT.
* measured on two occasions

Key to Table 3.15

SD	**Sibling Donor**
UD	**Unrelated donor**
Matched	**Matched at allelic level at HLA A*, B*,C*DR***
M/m	**mismatched**
NTD	**Non T depleted**
TD	**T depleted (ex vivo with 10-20 mg of Campath IH)**
PBSC	**Peripheral blood stem cells**
BM	**Bone Marrow**
TBI	**Total Body Irradiation (750cGy in a single fraction)**
Cy	**Cyclophosphamide 120mg/kg**
Bu	**Busulphan 8mg/kg**
Flu	**Fludarabine 150mg/m^2**
Mel	**Melphalan 140mg/m2**
Treo	**Treosulphan 10mg/m2/day for 3 days**
BEAM	**BCNU 300mg/m2, Cytarabine 200mg/m2/day for 4 days, Etoposide 200mg/m2/day for 4 days, Melphalan 140mg/m2**
Campath	**Campath 1H 50mg in 5 doses**

3.5 Chapter Discussion

Anti-CD28 antibody pre-treatment alone led to activation (increased CD69 expression) in PBMCs from 2 of 6 healthy donor samples. This heterogeneity of response may reflect an underlying polymorphism in these individuals increasing their sensitivity to anti-CD28-stimulated T cell activation. Such polymorphisms have been recently identified in the CTLA-4 gene leading to increased sensitivity to CD28-mediated T cell activation.[Djilali-Saiah *et al.* 2001;Huang *et al.* 2000;Ihara *et al.* 2001] The combination of OKT3 and anti-CD28 pre-treatment led to greater upregulation of several molecules important for alloantigen presentation than that seen with OKT3 alone but this additional benefit was not statistically significant.

OKT3 alone led to statistically significant increases in the expression of adhesion molecules (with the density of expression of CD11a increased by a factor of 1.6 and the percentage of T cells expressing CD54 increased by a factor of 8), which would potentiate the first stage of T-T antigen presentation. The density of HLA Class I molecules on T cells was increased by a factor of 4 by OKT3 treatment and the proportion of T cells expressing HLA Class II molecules (HLA DR) was increased by a factor of 5 from 8% to 40%. The increased expression of HLA molecules on T cells would facilitate autonomous antigen presentation. OKT3 pre-treatment also increased the proportion of T cells expressing CD86 by a factor of 4 (from 5% to 20% - above the threshold level of expression of co-stimulatory molecules need for effective tumour antigen presentation to T cells by AML blasts).[Whiteway *et al.* 2003]

Thus evidence has been presented for a molecular basis for the strategy of OKT3 pre-treatment of T cells to potentiation of their ability to present antigen.

OKT3 pre-treatment of PBMCs led to enrichment of the percentage of T cells within the PBMC pool with a concomitant decrease in the percentage of B cells and monocytes. A small proportion of monocytes persisted after 48 hours of OKT3 pre-treatment of PBMCs, which may have been able to augment T-T antigen presentation. The need for accessory APCells for maximum OKT3 mediated T cell activation has been demonstrated previously [van Lier *et al* 1989] and this raises the possibility of a dual antigen presentation pathways by an OKT3 pre-treated PBMC pool with T cells presenting antigen directly and antigen presentation via persistent monocytes/dendritic cells.

T-T antigen presentation has the theoretical advantage of non-presentation of myeloid-restricted antigens which when used in an MLR-based selective allodepletion protocol would not lead to the activation (or subsequent removal) of myeloid antigen-specific T cells within the donor T cell pool, potentially preserving T cell anti-myeloid responses within the donor cell pool that could exert a GvL effect in the treatment of myeloid malignancies.

The increase in the percentage of T cells expressing CD178 (from 5% to 20%) after OKT3 pre-treatment provides evidence for an additional molecular pathway that could potentiate the reduction in alloreactivity after CD69 based allodepletion. Alloreactive T cells in the donor T cell pool would receive an increased FAS ligand-mediated FAS signal that could result in alloreactive cell specific activation-induced cell death.

A concern regarding any strategy that exploits T-T antigen presentation is that responder cells are rendered anergic. The data presented in this chapter show that OKT3 pre-treatment of stimulator cells leads to increased responder cell activation (CD69 expression) and subsequent proliferation in the MLR in both the HLA-mismatched and matched setting. The strategy of OKT3 pre-treatment of stimulator cells lead to a much greater and consistent responder CD69 response that the cytokine pre-treatment strategy in HLA-matched pairs. The OKT3 pre-treatment strategy, in contrast to the cytokine strategy, represents a pathway to potentiate antigen presentation that is unlikely to be affected by stimulator (or responder) cytokine receptor polymorphisms.

The greatly increased responder CD69 expression after allostimulation in the MLR with OKT3 pre-treated stimulator cells suggests that a greater number of responder cells are activated than the expected frequency of alloreactive cells (1-5% in published data). It is unlikely that this vigorous responder cell response is due to a carry-over (and non-specific) effect of the OKT3 used to pre-treat the stimulator cells as;

1 The CD3 receptor needs to be cross-linked by OKT3 for effective T cell activation and this would not occur with OKT3 attached to stimulator cells of in solution carried over to responder cells.

2. The stimulator cells were vigorously washed following OKT3 pre-treatment to remove any excess OKT3 that could be carried over to responder cells.

3 OKT3-stimulated T cell activation leads to profound down-regulation of surface CD3 receptor expression which could be demonstrated after OKT3 pre-treatment of stimulator cells. Activated responder cells did not exhibit any significant reduction in CD3 expression (data not shown).

A more likely explanation for the increased frequency of activated responder cells in both HLA-mismatched and matched MLRs following OKT3 pre-treatment of stimulator cells is that OKT3 pre-treatment potentiates antigen presentation that results in the activation of T cells with low affinity for alloantigens as well as those with TCRs with high affinity for such antigens. This would not affect the selective allodepletion strategy (and might even lead to more effective allodepletion) but would not be desirable if the OKT3 pre-treatment strategy led to the activation (and subsequent removal) of donor T cells with cross-reactive TCRs, which share affinity for alloantigens and infective pathogen (e.g. viral) antigens.

Immunomagnetic selective allodepletion of HLA-matched $CD69^+$ responder cells was as efficient following OKT3 pre-treated allostimulation as that seen after the cytokine technique of potentiation of allostimulation. The strategy of OKT3 pre-treatment of stimulator cells led to responder CD69 upregulation (and proliferation) allowing selective removal of alloreactive cells in HLA-matched pairs that did not exhibit significant CD69 responses (or proliferation) with cytokine pre-treated stimulator cells. The use of the OKT3 pre-treatment strategy did not lead to a significant loss of proliferative responses to third party stimulators after selective allodepletion of first party responding cells. Thus the OKT3 pre-treatment strategy could permit alloantigen presentation and subsequent selective alloreactive cell depletion in HLA-matched pairs that do not exhibit a positive read-out in the cytokine-modified MLR. The S:R ratio data suggest that OKT3 pre-treatment of stimulator cells might lead to effective presentation of alloantigens (and subsequent responder cell CD69 expression) with S:R ratios less than 1:1 which might enable selective allodepletion when stimulator cells are in short supply.

No data have yet been published regarding the positive or negative predictive value of proliferative responses in the OKT3 pre-treated HLA-matched MLR. Responder proliferation in the MLR correlates most closely with responder

$CD4^+$ responses but other measures of responder cell activation (e.g. $CD8^+$ IFN-γ secretion by flow cytometric methods or in ELISpot assays) could also be studied to define cellular responses to alloantigens before and after selective allodepletion following the use of OKT3 pre-treated stimulators. Abrogation of alloresponses following selective allodepletion could also be confirmed with the skin explant assay although obtaining skin biopsies from recipients in donor-recipient pairs has proved very difficult in practice.

A small and heterogeneous group of HLA-matched pairs were studied, and interpretation of the data regarding proliferation in the OKT3-modified MLR and acute GvHD must therefore be extremely cautious.

3 haematopoietic grafts were T cell depleted with Campath antibody *ex vivo*. 7 transplants utilised full intensity conditioning (6 with total body irradiation) and 7 reduced intensity conditioning. Additionally the source of haematopoietic stem cells varied, (11 PBSCs and 4 bone marrow). All of these factors have profound bearing on the risk of developing acute GvHD. Thus it is difficult to draw meaningful conclusions from data from the group regarding any association between proliferation in the MLR with OKT3 pre-treated stimulators and the occurrence and/or severity of GvHD.

In the data presented here, of 7 pairs with full intensity conditioning, 6 had an OKT3 RRI>/= 50% and of these 6, 4 developed >/= acute grade 2 GvHD (PPV 66%) and the single pair with an OKT3 MLR RRI<50% did not.

Of the 6 pairs in which reduced intensity conditioning was used, if a similar cut off level of 50% for OKT3 MLR RRI is used then one of the 3 pairs with RRI>50% developed for >/= acute grade 2 GvHD (PPV 33%) whereas the 2 of the 3 pairs with RRI<50% did not (NPV 66%).

To reliably establish the positive predictive and negative predictive value of the OKT3 modified MLR, a larger series of non T cell depleted transplant pairs should be studied, homogeneous for recipient conditioning, donor matching and stem cell source.

The original data published by Bishara describing the cytokine modified MLR detailed 16 non-T cell depleted, HLA-matched, full intensity conditioning sibling transplants, in which a cut off level of RRI of 5% gave a PPV of 66% (4 of 6 with RRI>5% developed acute GvHD) and NPV of 90% (1 of 10 with RRI<5% developed >/= grade 2 acute GvHD). Koh reported cytokine modified MLRs in 8 HLA-matched pairs (6 sibling and two unrelated, one of which was T depleted.)

All patients received a TBI based conditioning regimen. 4 of these had a cytokine MLR RRI>5% and were evaluable for ≥ grade 2 aGvHD. In this series the cytokine MLR RRI >5% had both a PPV of 50% and NPV of 75%.

In the data presented here, of the seven pairs with full intensity conditioning, 6 had RRI>/= 5% (there was a higher proportion of unrelated donor pairs in these data than in Koh's series) and of these 6, 4 patients developed ≥ grade 2 aGvHD (PPV 66%) and the single pair with a cytokine RRI<5% did not. This is in keeping with the published data.

All of the 6 pairs in which reduced intensity conditioning was used had cytokine MLR RRIs>5%, but only one recipient developed for ≥ grade 2 aGvHD. It is likely that a higher cut off level for cytokine MLR RRI is a better predictor of acute GvHD following reduced intensity conditioning e.g. if a cut off of 80% for cytokine MLR RRI is used then none of the 5 pairs with RRI<80% developed for ≥ grade 2 aGvHD (NPV 100%) whereas the single pair with RRI>80% did.

Data from pair 13 (DRB1*04 mismatch, in which failure of proliferation in cellular assays is well described despite frequent severe GvHD) were excluded from the above discussion.[Mickelson *et al.* 1993;Mickelson *et al.* 1996]

Chapter 4 Retention of Antiviral Responses After Allodepletion

4.1 Introduction

CMV infection post AHSC transplant is an important cause of morbidity and mortality and has considerable economic impact.[Squifflet and Legendre 2002] A significant number of adults who undergo AHSCT have had serologically detectable past exposure to CMV. In Meyer's series from 1986 as many as 70% of CMV seropositive transplant recipients developed clinically significant CMV infection and CMV disease was the commonest cause of death in such patients.[Meyers *et al.* 1986] Prophylactic treatment with ganciclovir or aciclovir has only partially reduced CMV infection post-AHSCT and has the theoretical disadvantage of encouraging drug-resistant viral strains.[Prentice *et al.* 1994] The strategy of monitoring patients for viral shedding by the detection of viral genome by (real-time) PCR reduces CMV infection and death in the first 100 days post AHSCT but may have the effect of postponing infection to a later stage post transplant.[Ljungman 1998] Such pre-emptive treatment is associated with adverse effects especially (reversible) neutropenia that often requires cessation of treatment. The crucial importance of T cell mediated immunity in controlling CMV disease after allogeneic transplant is underlined by the increased rate of CMV pneumonitis reported after non-selectively T cell depleted AHSCT.[Nguyen *et al.* 1999] A lower incidence of CMV pneumonitis was reported in CMV seropositive recipients of non-T cell depleted AHSCT from CMV seropositive donors as long ago as 1987.[Grob *et al.* 1987] Moreover there is evidence that grafts containing T cells from CMV positive donors may confer a survival advantage under certain circumstances to recipients at high risk of CMV disease. The recent European Bone Marrow Transplant metafile analysis demonstrated that CMV seropositive patients receiving grafts from CMV-seropositive HLA-matched sibling donors had the same survival as patients transplanted with seronegative donors. However, CMV-seropositive stem cell transplant recipients receiving grafts from CMV-seropositive unrelated donors had significantly better 5-year overall survival and reduced transplant-related mortality. Although this benefit was not found to be directly linked to CMV disease post transplant it was independent of all other variables. Furthermore in such patients with CML, T cell depletion abrogated the beneficial effect of donor

CMV status, suggesting that the effect is mediated through transfer of donor T cell immunity.[Ljungman *et al.* 2003a]

The $CD8^+$ CTL response is of critical importance for control of CMV infection and it has been estimated that between 1 and 5% of CTLs in healthy CMV seropositive individuals have specificity for CMV.[Gillespie *et al.* 2000]

In controlling CMV disease $CD8^+$ responses are believed to be the main effector arms of the adaptive immune system although they are critically dependent on $CD4^+$ T cell help.[Gamadia *et al.* 2003] In the absence of $CD4^+$ help there may be persistence of virus despite the presence of $CD8^+$ CMV-specific CTLs as the latter become anergic.[Zajac *et al.* 1998]

EBV-related PTLD is another cause of significant morbidity and mortality after allogeneic haematopoietic stem cell transplantation, usually a monoclonal expansion of B cells with predominantly latent type infection with the EBV virus.[Tanner and Alfieri 2001] EBV-specific CTL responses are of paramount importance in the control of EBV-PTLD, and infusion of very small numbers of donor-derived EBV-specific CTLs are very effective at suppressing EBV-related PTLD.[Heslop *et al.* 1994]

Non-selective ex vivo T cell depletion techniques using anti-T cell antibodies with broad specificities such as the anti-CD52 antibody Campath removes both $CD8^+$ and $CD4^+$ CMV-specific cells and slows the recovery of CMV-specific CTLs (identified by tetramer and ELISpot assays) post-AHSCT.[Aubert *et al.* 2001;Chakrabarti *et al.* 2002b]

A major advantage of selective alloreactive cell depletion would be to leave the donor $CD8^+$and $CD4^+$ T cell pool largely intact, which would (in the case of a CMV and EBV seropositive donor) contain CMV and EBV-specific $CD8^+$ and $CD4^+$ cells; able to confer active immunity against CMV infection and EBV-PTLD.

However it has been previously reported that among the population of T cells specific for allogeneic MHC, there is a mix of naïve and memory cells. [Ashwell *et al.* 1986b] It has also been shown that TCRs are broadly cross-reactive; and many T cells specific for environmental antigens also cross-react with foreign MHC. [Matzinger 1994]

On the basis of these data, it could be hypothesized that T cell allodepletion might result in a reduction of the number of T cells specific for viral antigens. The degree of reduction (if any) seen would depend on the number of T cells

possessing TCRs cross-reactive for alloantigens and for viral antigens and also on the relative strengths of TCR/MHC-Antigen interactions of such T cells.

The preservation of proliferative responses to CMV and candida antigens in cell fractions depleted of cells alloreactive to haploidentical stimulators has been reported after allodepletion based on expression of CD25. Preservation of CTLp frequencies against CMV and EBV infected cells has also been demonstrated in an HLA-mismatched in vitro model utilising CD25-mediated immunotoxin based allodepletion. [Montagna *et al.* 1999]

Very recently this approach was refined by the use of haploidentical recipient EBV-transformed LCLs as stimulators to activate donor alloreactive T cells. In IFN-γ ELISpot assays, $CD8^+$ T cell responses to adenovirus and CMV were preserved following allodepletion and the latter was confirmed with the use of HLA-A*0201-NLV-tetramers. Donor anti-EBV responses were also partially retained. [Amrolia *et al* 2003a]

Thus there is good evidence to support the conclusion that with TCRs cross-reactive with antigens on such pathogens and stimulator non-self HLA molecules are not present at significant frequencies, or that they are not identified and removed by the CD25-mediated allodepletion technique.

No data have been published regarding preservation of antiviral responses following CD25-mediated allodepletion in the HLA-matched setting.

A very limited amount of data concerning the preservation of anti-CMV responses after selective allodepletion based on CD69 expression has been published and only in abstract form. Nonn et al have reported enrichment of CMV reactive cell frequencies (assayed by pp65-stimulated IFN-γ ELISpot) in the $CD69^{dim}$ responder cell fraction in HLA-mismatched MLRs. This observation would be consistent with T cells sharing TCR affinity for both alloantigens (perhaps with weak affinity) and CMV antigens.[Nonn *et al* 2003a] Schumm et al have reported preservation of CMV- and adenoviral–specific $CD8^+$ cells (assayed by IFN-γ ELISpot) following CD69 allodepletion in some, (but not all) HLA-matched donor-recipient pairs using the cytokine modified MLR to potentiate PBMC mediated allostimulation. This observation would be consistent with the existence of T cells bearing TCRs cross-reactive for stimulator mHags and CMV antigens.[Schumm *et al* 2003]

The work detailed in this chapter seeks to investigate whether antiviral activity is preserved after CD69-mediated allodepletion in a short series of pilot experiments in the HLA-mismatched setting, and in a more detailed examination in the HLA-matched setting.

CD69-based selective allodepletion (by immunomagnetic sorting) was used to remove alloreactive cells from responder cells in totally HLA-mismatched donor/recipient pairs and in HLA A, B, C and DR-matched (sibling and unrelated) donor/recipient pairs. Pairs were selected where the donor was known to be CMV and EBV (EBNA) IgG^+. HLA-matched pairs were selected with a positive cytokine-modified MLR (RRI>5%). Both the cytokine-modified MLR and the OKT3 pre-treatment techniques were used to potentiate allostimulation in the HLA-matched pairs.

CMV-reactive CTLs were enumerated before and after selective allodepletion by CMV-peptide-specific IFN-γ ELISpot assay and by CMV-peptide HLA A*0201 tetramer analysis. EBV-reactive T cells were enumerated by IFN-γ ELISpot before and after selective alloreactive cell depletion in the HLA-matched setting.

4.2 Aims of Experiments Described in this Chapter

1. To investigate the retention of CMV- and EBV- specific CTLs after selective allodepletion (based upon CD69 expression) in HLA-mismatched and HLA-matched pairs.
2. To compare retention of CMV- and EBV- specific CTLs after selective allodepletion (based on CD69 expression) in HLA-matched pairs using both the cytokine and the OKT3 techniques for potentiating allostimulation.

4.3 Materials and Methods

4.3.1 CMV and EBV serology

CMV IgG and EBV EBNA IgG serological status was measured by ELISA in the Department of Virology, Royal Free Hospital or at accredited haematopoietic stem cell donor centres. All cells used were from peripheral blood samples given with written informed consent.

4.3.2 CMV- and EBV-peptides used in IFN-γ ELISpot Assays

Peptide epitopes within the CMV matrix protein pp65 have been identified as important targets for CMV–specific $CD8^+$ cells, and $CD8^+$ CTLs specific to pp65 derived peptides persist at high frequencies in individuals exposed to CMV virus.[Longmate *et al.* 2001;Wills *et al.* 1996a] A number of different pp65 derived peptides were thus selected with different HLA Class I restrictions which permitted the measurement of CMV-specific CTL responses in individuals of several different HLA Class I types.

To assess the frequency of cellular responses to EBV, the HLA-A*0201-restricted epitope GLCTLVAML derived from the EBV lytic protein BMLF1 was chosen. This lytic EBV antigen is recognized by a relatively large fraction of EBV-specific $CD8^+$ T cells in HLA-A*0201 donors in various patho-physiological situations,[Lim *et al.* 2000a;Steven *et al.* 1997] and is restricted to a common HLA-A type (and would facilitate concurrent experiments measuring CMV- and EBV-CTL responses in individual samples).

The ELISpot assay was performed in an identical fashion for CMV- and EBV-peptide-stimulated responses (Chapter 2.6). The negative control for the EBV-peptide-stimulated ELISpot assay was 'no peptide'. Two negative controls for CMV-peptide-stimulated ELISpot assays were performed; 'no peptide' and a 'dummy peptide' (a CMV pp65-derived peptide restricted to an HLA Class I type not possessed by the individual). The purpose of the dummy peptide control in this setting was to ensure that the CMV-peptides only elicited responses in the expected HLA- restricted fashion and did not cause non-specific stimulation of $CD8^+$ cells.

Virus	Amino Acid Sequence	HLA Class I Restriction	Abbreviation	Structural site
CMV	NLVPMVATV	A*0201	NLV	CMV pp65 (495-503)
CMV	TPRVTGGGAM	B*0702	TPR	CMV pp65 (417-426)
CMV	DANDIYRIF	B*0801	DAN	CMV pp65 (516-524)
CMV	IPSINVHHY	B*35	IPS	CMV pp65
CMV	YSEHPTFTSQY	A*0101	YSE	CMV pp65
EBV	GLCTLVAML	A*0201	GLC	EBV Lytic protein BMLF1

Table 4.1 Human CMV- and EBV-peptides used for IFN-γ ELISpot assays.

4.3.3 CMV NLVPMVATV-HLA A*0201 Tetramer Assay

Details of the staining protocol and the flow cytometric analysis (including gating strategy) are given in Chapter 2.2.3.

4.3.4 Validation of CMV-Peptides and CMV IFN-γ ELISpot Assay

Individual	HLA Class I typing	CMV stimulatory peptide	CMV dummy Peptide
1	A*0301,2601;B*0702,0801	TPR	NLV
2	A*2,25; B*14,58	NLV	TPR
3	A*0101,24;B*27,62	YSE	NLV
4	A*1,2; B*63,68	NLV	TPR
5	A*0201;B*15,39	NLV	TPR

Table 4.2 Healthy controls used for validation of CMV stimulatory peptides.

4.3.5 Experimental Design

Allostimulation of HLA-mismatched pairs was performed in a one-way standard MLR with cells from the CMV^+ donor acting as responders. Allostimulation was confirmed by measuring expression of CD69 on live responder cells and by a incorporation of tritiated thymidine in a standard 5 day one way MLR (Chapter 2.3.2). Selective allodepletion was performed using anti-CD69–FITC antibody (BD), anti-FITC microbeads and AS single-use columns and the VarioMACS magnet (all Miltenyi). Efficiency of allodepletion was confirmed by using allodepleted cells in a secondary MLR with original (first party) stimulators and with HLA-mismatched third party stimulators, (Chapter 2.4). The frequency of CMV-responding cells (by both IFN-γ ELISpot and by PE conjugated NLV-HLA A*0201 tetramer) and EBV responding cells (by IFN-γ ELISpot) was determined both in unmanipulated live responder cells and selectively allodepleted live responder cells.

The schema was followed for the HLA-matched pairs with the MLR modified by the pre-treatment of stimulators with either OKT3 or cytokines (as described in Chapter 2.3.3 and 2.3.4).

4.3.6 HLA Typing and CMV/EBV-Peptides used

Pair	Stimulator HLA typing	Responder HLA Typing	CMV-peptide	Dummy peptide
1	A*0201, *24; B*5101, *4405; C*0202, *1502; DRB1*04, *11	A*0301,2601; B*0702,0801 HLA C not done DRB1*1501, *0301	TPR	NLV
2	A*0201, *24; B*0702, *1401; C*07, *08; DRB1*0401, *0701	A*0101; B*0702, *3701; C*06, *07; DRB1*0404, *0701	TPR	NLV
3	A*0101; B*0702, *3701; C*06, *07; DRB1*0404, *0701	A*0201, *24; B*0702, *1401; C*07, *08; DRB1*0401, *0701	NLV	YSE
4	A*0101,*2401; B*0702,*0801; C*07, *07; DRB1*15, *03	A*0201 HLA B and Class II not assessed	Not done	Not done
5	A*2402,*3301; B*4405,*3508 C*02, *04; DRB1*16, *13	A*0201 HLA B and Class II not assessed	Not done	Not done

Table 4.3 Stimulator and Responder HLA types and CMV-peptides used in ELISpot assays in HLA-mismatched pairs.

Pair	Stimulator HLA typing	Responder HLA Typing	ELISpot Peptide		
			CMV		EBV
			Stimulatory Peptide	Dummy Peptide	
1	A*0201, *302; B*52, *40;C*15, *12; DRB1*1502, *16	HLA-matched SD	NLV	TPR	GLC
2	A*0201, *68; B*51, *0702; C*15, *07; DRB1*1501/13021	HLA-matched SD	NLV	YSE	GLC
3	A*0201, *33;B*58, *35 C*03, *04; DRB1*1601, *0301	HLA-matched SD	NLV	TPR	GLC
4	A*0201, *30;B*0702, *44; C*07, *05; DRB1* 1401, *0810	HLA-matched SD	NLV	YSE	GLC
5	A*0101; B*0801; C*0701; DRB1* 0301	HLA -matched UD	DAN	NLV	Nd
6	A*0201, *24; B*0702, *14;C*07, *08; DRB1*0401, *0701	HLA -matched UD	NLV	YSE	Nd
7	A*0201, *0101; B*08, *04; C*03, *07; DRB1*0101, *0301	HLA -matched UD	NLV	TPR	GLC
8	A*0201, *29; B*08, *15; C*07, *03; DRB1*0301, *13	HLA -matched UD	NLV	TPR	GLC
9	A*0101; B*0702, *37; C*06, *07; DRB1*0404, *0701	Single HLA A -mismatched UD	TPR	NLV	Nd
10	A*0201: B*44,35;C*04,05 DRB1*1501, *1401	HLA A and C -mismatched UD	NLV	TPR	Nd
11	A*0201, *0301; B*35, *37; C*04, *06, DRB1*0404, *1303	Single DRB1 -mismatched UD	NLV	TPR	Nd

Table 4.4 Stimulator and Responder HLA types, CMV-and EBV-peptides used in ELISpot assays in HLA-matched pairs. SD =sibling donor, UD= Unrelated donor, nd= not done.

4.4 Results

4.4.1 Validation of HLA Class I restriction of CMV-Peptides

The specific binding of the HLA A*0201-restricted peptide NLV to HLA A*0201 was confirmed in a T2 binding assay (Chapter 2.11). The MHC stabilisation efficiency (MSE) of NLV was 84% whereas the B*0702-restriced CMV pp65 derived peptide TPR gave an MSE of 2% (Figure 4.1).

Cytotoxicity assays using PKH-26 dye labelled T2 target cells and healthy donor CTLs were performed in duplicate, (described in detail in Chapter 2.10).

Specific killing of PKH-26 dye labelled T2 target cells was significantly greater with NLV peptide pulsed T2 target cells than with untreated T2 cells with both unselected and CMV whole antigen-pulsed healthy HLA A*0201$^+$ CMV IgG$^+$ donor CTLs. Specific killing of untreated PKH-26 dye labelled T2 target cells was significantly greater with CMV whole antigen -pulsed healthy HLA A*0201$^+$ CMV IgG$^+$ donor CTLs than with unselected healthy HLA A*0201$^+$ CMV IgG$^+$ donor CTLs. Although greater NLV peptide pulsed T2 target cell killing was seen with CMV whole antigen-pulsed healthy HLA A*0201$^+$ CMV IgG$^+$ donor CTLs than with unselected donor CTLs this was not statistically significant. (Figure 4.2)

4.4.2 Validation of the NLV-HLA A*0201 Tetramer Assay

10^6 PBMCs from an HLA A*0201$^+$ B*0702$^-$ CMV IgG$^+$ healthy subject were incubated for 30 minutes with 10μg NLV or TPR CMV-peptide. PBMCs were then centrifuged for 10 minutes at 200g and resuspended at 10^6 cells/ml in complete medium, and stained with NLV-HLA A*0201 tetramer (Chapter 2.2.3). The prior addition of NLV peptide to PBMCs from an HLA A*0201$^+$ B*0702$^-$ CMV IgG$^+$ healthy subject prevented the binding of NLV-HLA A*0201 tetramer to CD3$^+$CD8$^+$ CMV-specific CTLs whereas the addition of the non-HLA A*0201-restricted CMV-peptide TPR did not (Figure 4.3).

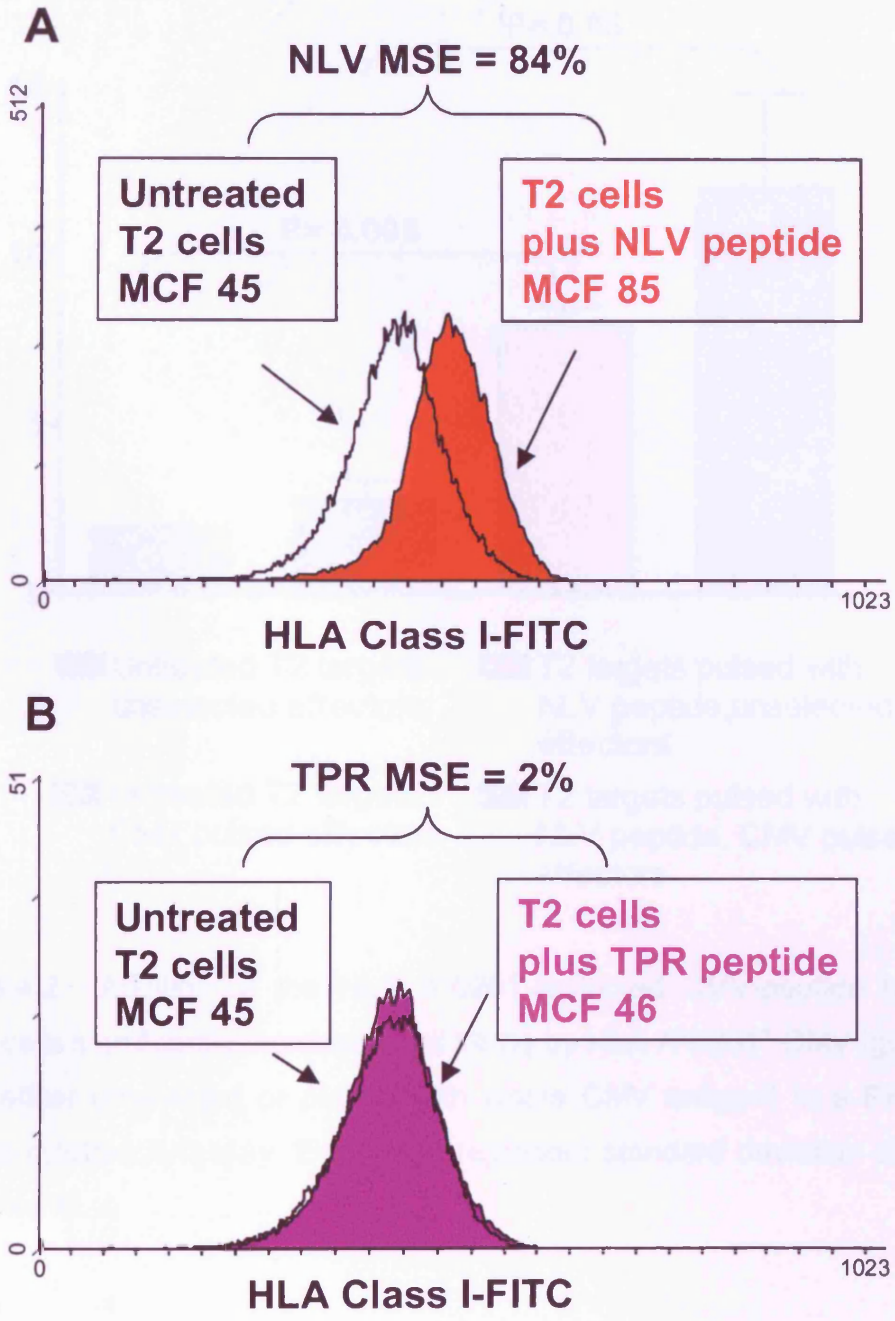

Figure 4.1 T2 binding assays and MHC Stabilisation Efficiency (MSE).

(A). NLV CMV-peptide

(B). TPR CMV-peptide

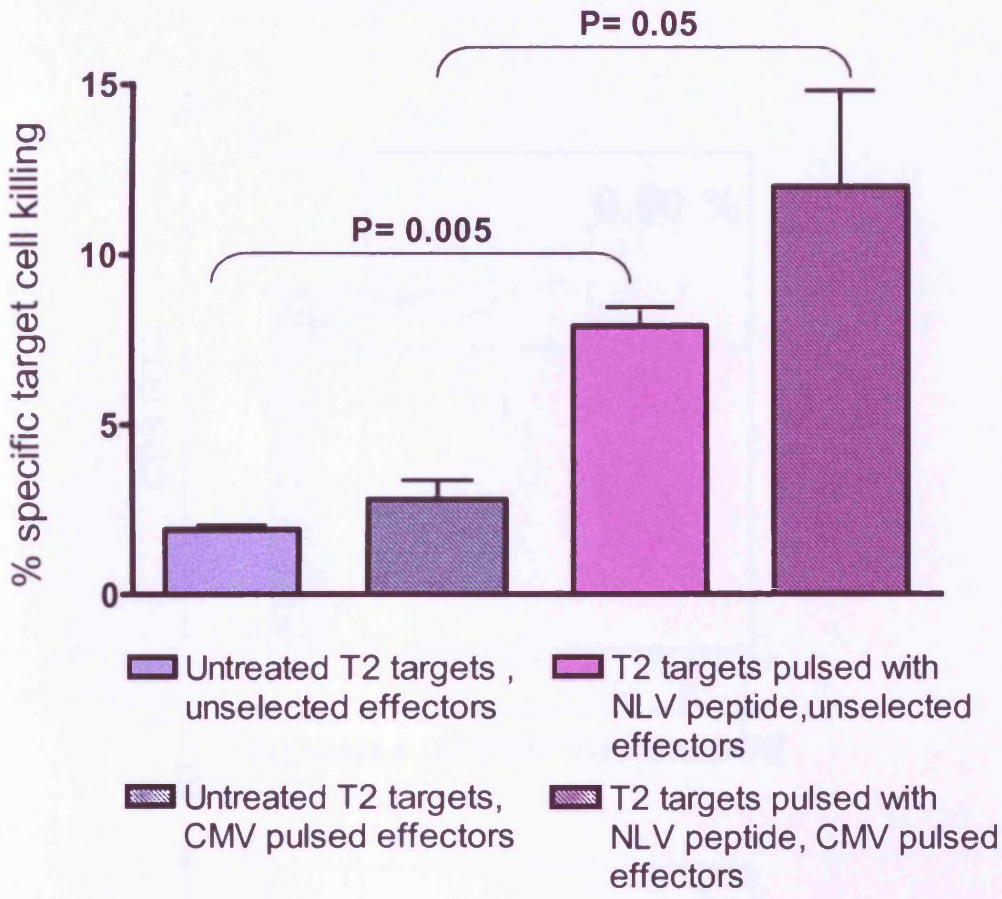

Figure 4.2 Addition of the HLA A*0201-restricted CMV-peptide NLV to T2 target cells significantly increased cell killing by HLA A*0201$^+$ CMV IgG$^+$ effector cells (either unselected or pulsed with whole CMV antigen) in a PKH-26 dye release cytotoxicity assay. Error bars represent standard deviation of duplicate experiments.

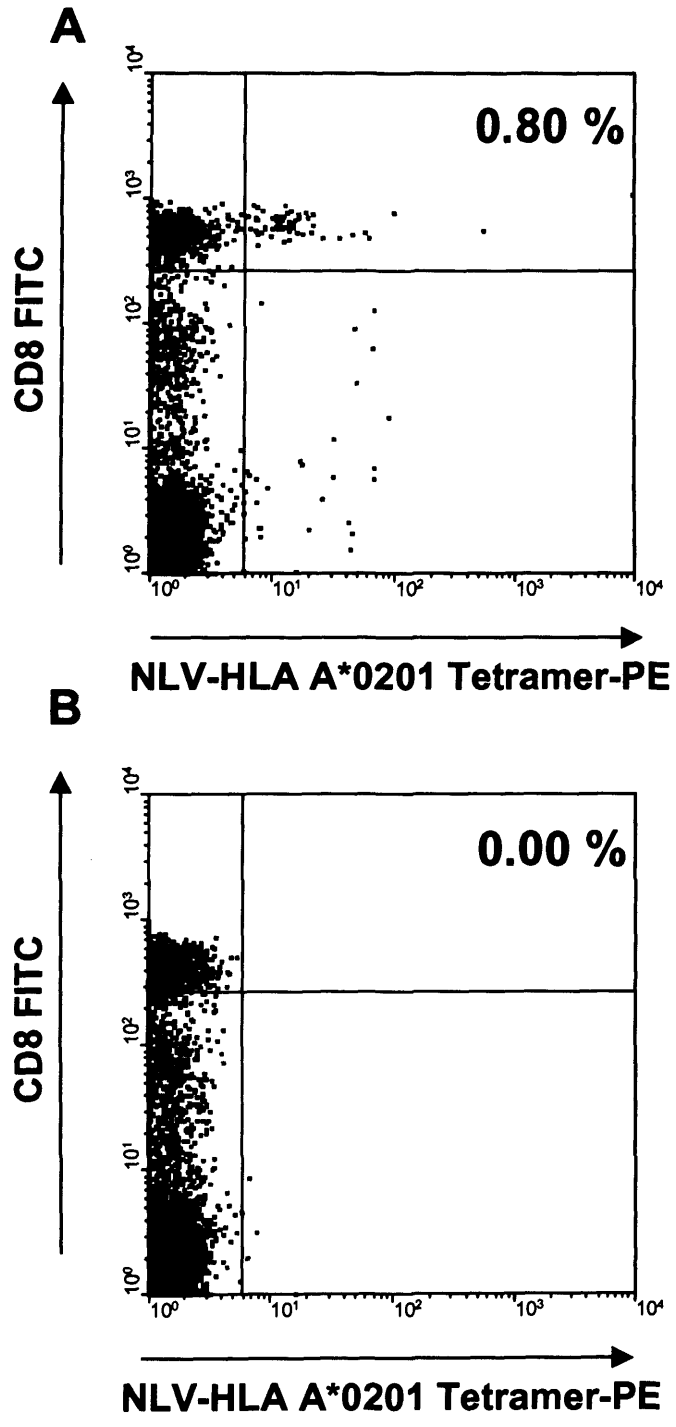

Figure 4.3 NLV-HLA A*0201 tetramer binding to CMV-specific CTLs in an HLA A*0201$^+$ CMV IgG$^+$ subject. The figures shown are percentage CD3$^+$CD8$^+$ Tetramer $^+$ cells

(A). Tetramer binding is seen with untreated PBMCs

(B). Tetramer binding is inhibited by the addition of exogenous NLV peptide prior to tetramer staining.

4.4.3 CMV-CTL Responses in Normal Individuals

The baseline frequencies of CMV-specific CTLs were determined in 5 healthy CMV IgG$^+$ individuals using NLV-HLA A*0201 tetramer staining (Chapter 2.2.3), (for 3 HLA A*0201$^+$ individuals) and CMV-peptide-stimulated IFN-γ ELISpot assay (Chapter 2.6). (Details of stimulatory and dummy (control) peptides used for ELISpot assays are given for each individual in Table 4.2)

Control Subject	HLA Class I Typing	NLV-HLA A*0201-Tetramer$^+$ frequency	CMV-Peptide IFN-γ ELISpot reactive cells (mean+/-sd)
1	A*0301,2601;B*0702,0801	*nd*	*0.37 +/- 0.02*
2	A*2,25; B*14,58	*5.0*	*3.47 +/- 0.50*
3	A*0101,24;B*27,62	*nd*	*0.05 +/- 0.02*
4	A*1,2; B*63,68	*0.3*	*0.13 +/- 0.03*
5	A*0201;B*15,39	*1.3*	*0.77 +/- 0.24*

Table 4.5 Frequency of CMV-specific CTLs in normal individuals. Figures given are percentage CD3$^+$CD8$^+$ Tetramer$^+$/ELISpot reactive cells. nd= not done

4.4.4 Allodepletion in HLA-Mismatched Pairs

	Pre-Depletion Responder CD3$^+$CD69 expression, %	Depletion efficiency of CD3$^+$CD69$^+$ cells, %	Post-Depletion	
			First Party Proliferation*	Third Party Proliferation*
CD3$^+$	*6.0+/-4.0*	*91+/-7.0*	*1.9+/-3.8*	*69+/-31*
CD3$^+$CD4$^+$	*4.3+/-2.5*	*94+/-6.2*		
CD3$^+$CD8$^+$	*4.8+/-3.4*	*86+/-11*		

Table 4.6 Allostimulation, depletion efficiency and residual first party and third party proliferation after depletion of alloreactive CD69$^+$ cells in HLA-mismatched stimulator-responder pairs. All values are given as mean percentage +/-sd (above autologous controls). *= percentage of pre-depletion value.

4.4.5 Allodepletion in HLA-Matched Pairs

OKT3 MLR	Pre-Depletion		Depletion efficiency of $CD3^+CD69^+$ cells, %	Post-Depletion	
	Responder $CD3^+CD69$ expression	RRI		RRI,%	Third Party Proliferation *
$CD3^+$	*25+/-12*	*72+/-49*	*96+/-3*	*1+/-2*	*94+/-47*
$CD3^+CD4^+$	*29+/-13*		*94+/-9*		
$CD3^+CD8^+$	*15+/-9*		*92+/-15*		

Table 4.7 OKT3 pre-treated allostimulation, depletion efficiency and residual first party and third party proliferation after depletion of alloreactive $CD69^+$ cells in HLA-matched stimulator-responder pairs. All values are given as mean percentage +/-sd (above autologous controls). *= percentage of pre-depletion value.

Cytokine MLR	Pre-Depletion		Depletion efficiency of $CD3^+CD69^+$ cells, %	Post-Depletion	
	Responder $CD3^+CD69$ expression	RRI		RRI,%	Third Party Proliferation *
$CD3^+$	*5+/-7*	*44+/-35*	*82+/-16*	*1+/-2*	*101+/-90*
$CD3^+CD4^+$	*6+/-8*		*90+/-10*		
$CD3^+CD8^+$	*3+/-4*		*90+/-11*		

Table 4.8 Cytokine pre-treated allostimulation, depletion efficiency and residual first party and third party proliferation after depletion of alloreactive $CD69^+$ cells in HLA-matched stimulator-responder pairs. All values are given as mean % +/-sd (above autologous controls). *= percentage of pre-depletion value.

4.4.6 Retention of CMV responses in HLA-Mismatched Pairs

The frequency of $CD3^+CD8^+$ NLV-HLA A*0201 $tetramer^+$ cells after selective allodepletion of HLA-mismatched pairs 4 and 5 was not significantly reduced when compared to baseline pre-depletion values ($p=0.10$ in a paired two-tailed Student's t-test). The post-allodepletion frequency of $CD3^+CD8^+$ NLV-HLA A*0201 $tetramer^+$ cells was 88%+/-6.6% of the pre-depleted frequency.

Functional CMV-specific cell frequencies (measured by CMV-peptide-stimulated IFN-γ ELISpot assay) were also not significantly reduced after selective allodepletion of HLA-mismatched pairs 1,2 and 3 ($p=0.88$ in a paired two-tailed Student's t-test). Following allodepletion of these pairs the frequency of CMV reactive cells by IFN-γ ELISpot was 114%+/-35% of the frequency in the pre-depleted cells (Figure 4.4).

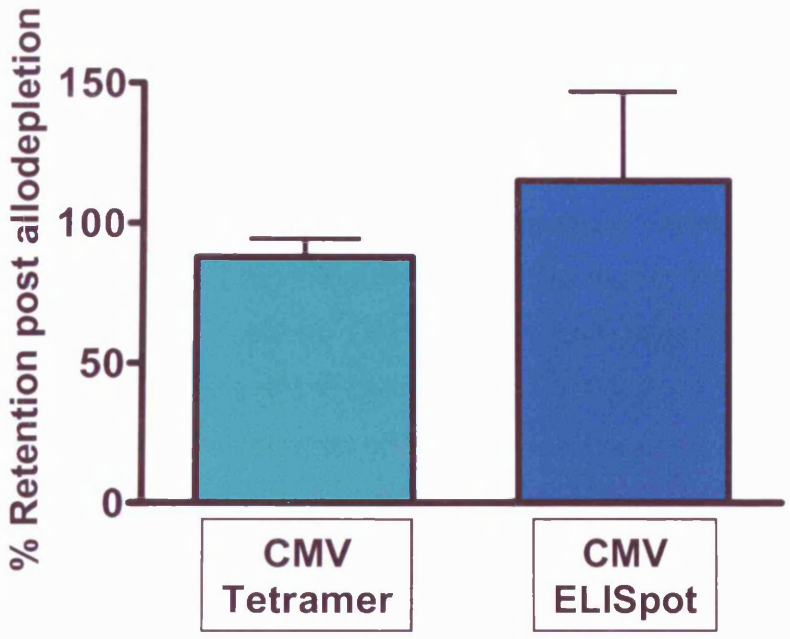

Figure 4.4 Retention of NLV-HLA A*0201 tetramer$^+$ cells (n=2) and CMV-peptide IFN-γ ELISpot-reactive cells (n=3) after selective allodepletion in 5 HLA-mismatched pairs. Error bars represent standard deviation. Percentage retention is frequency of $CD3^+CD8^+$ Tetramer$^+$/ELISpot reactive cells post-depletion x100/ frequency of $CD3^+CD8^+$ Tetramer$^+$/ELISpot reactive cells pre-depletion.

4.4.7 Retention of NLV-HLA A*0201 tetramer$^+$ Cells in HLA-Matched Pairs

All matched pairs studied for retention of NLV-HLA A*0201 tetramer$^+$ cells were HLA A*0201$^+$ The frequency of NLV-HLA A*0201 tetramer$^+$ cells (expressed as percentage of $CD3^+CD8^+$ cells) in responder cells after 72 hours of allostimulation was not significantly different to that of baseline, unmanipulated responder cells using either OKT3 pre-treated stimulators or cytokine pre-treated stimulators (data not shown).

After effective selective allodepletion of 7 HLA-matched pairs $CD3^+CD8^+$ NLV-tetramer$^+$ cell frequency post-depletion was 77%+/-20% of the pre-depletion frequency utilising the OKT3 pre-treated MLR and 98%+/-26% utilising the cytokine-modified MLR, (Figure 4.5). Post-depletion frequencies were not significantly different to pre-depletion frequencies in a paired, two-tailed Student's t-test ($p=0.06$ and $p=0.7$) although a trend was seen for lower $CD3^+CD8^+$ NLV-tetramer$^+$ frequency post-depletion following the OKT3 allostimulation technique. Representative dot-plots for an HLA-matched sibling pair and an HLA-matched unrelated donor-recipient pair are shown in Figure 4.6. In 6 of the 7 HLA-matched individual pairs tested the $CD3^+CD8^+$ NLV-tetramer$^+$ frequency post-depletion was lower than the pre-depletion value using the OKT3 allostimulation technique whereas this was the case in only 3 of the 7 pairs tested using the cytokine allostimulation technique (Figure 4.7). No significant difference was seen in the percentage retention of $CD3^+CD8^+$ NLV-tetramer$^+$ cells following allodepletion using the OKT3 or cytokine allostimulation techniques ($p=0.12$ in an unpaired two-tailed Student's t-test).

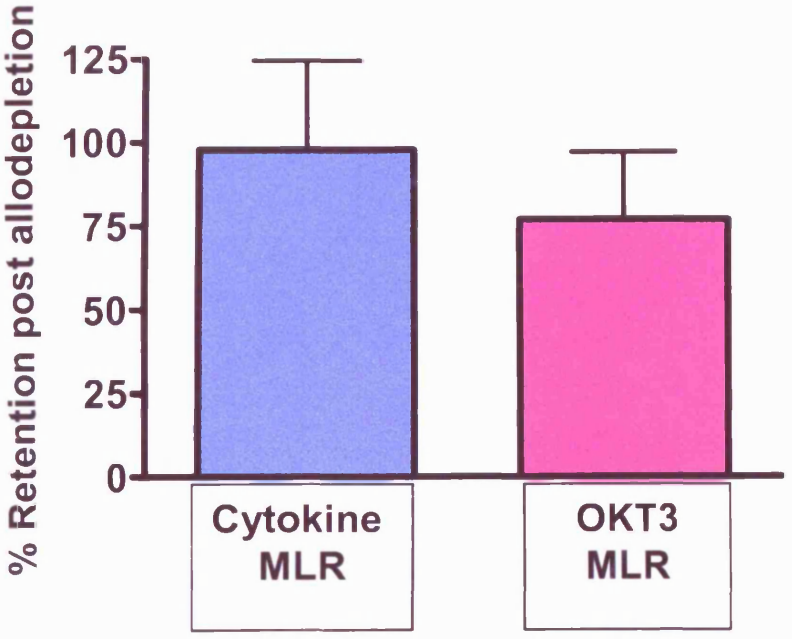

Figure 4.5 Percentage retention of NLV-HLA A*0201 tetramer$^+$ cells after selective allodepletion in 7 HLA-matched pairs utilising the cytokine and the OKT3 allostimulation techniques. Error bars represent standard deviation. Percentage retention is frequency of $CD3^+CD8^+$ tetramer$^+$ cells post-depletion x100/ frequency of $CD3^+CD8^+$ tetramer$^+$ cells pre-depletion.

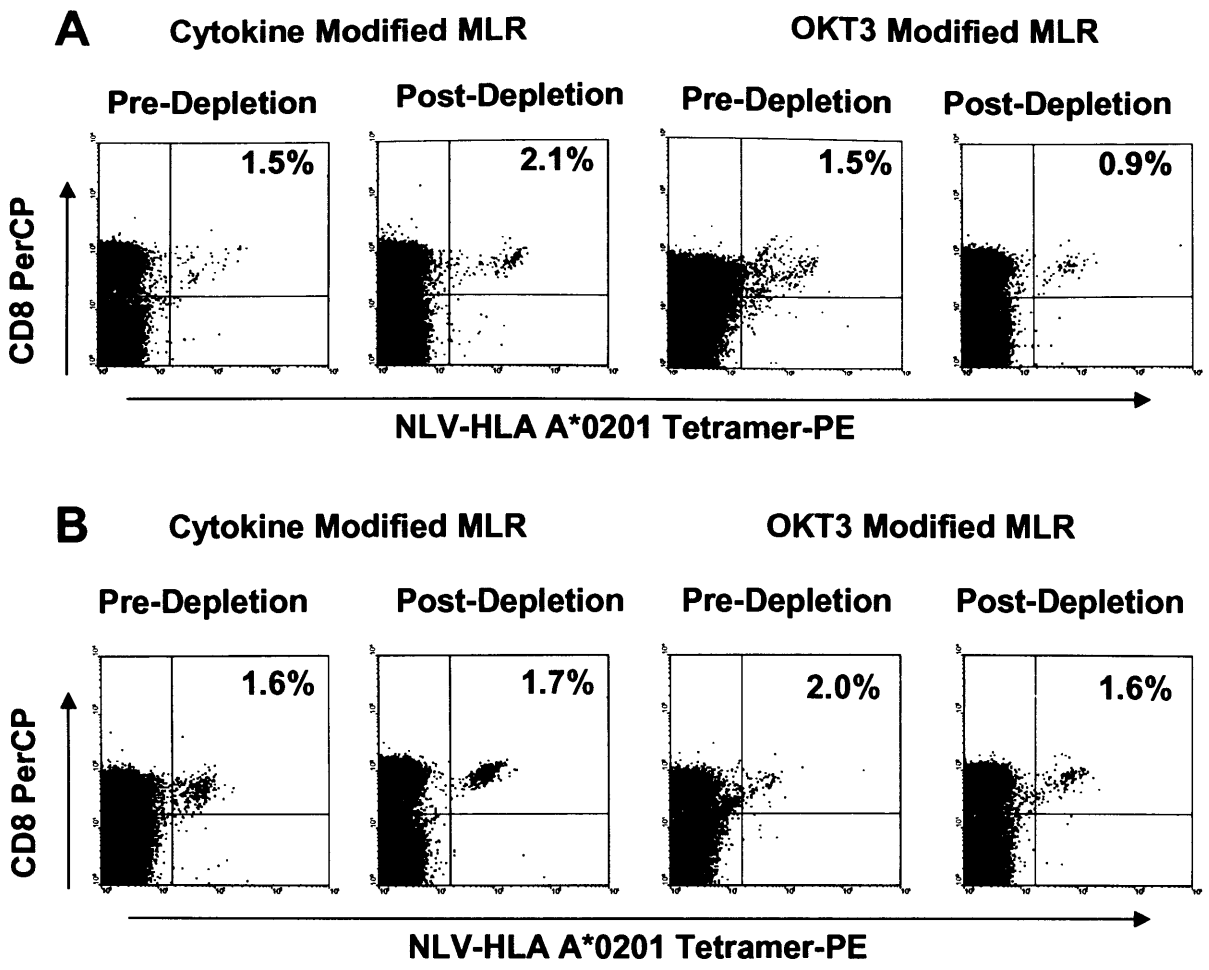

Figure 4.6 See Over for Figure Legend

Figure 4.6 CMV NLV-HLA A*0201 tetramer$^+$ cells are retained after depletion of alloreactive cells in the MLR. Example dot plots for;
(A). An HLA A, B, C and DR matched sibling pair (matched pair 2)
(B). An HLA A, B, C and DR matched unrelated pair (matched pair 7)
The figures shown were generated in WinMDI following the gating strategy described in Chapter 2.2.3 Figures shown are for tetramer binding cells, expressed as percentage of $CD3^+CD8^+$ cells in the responder cell region.

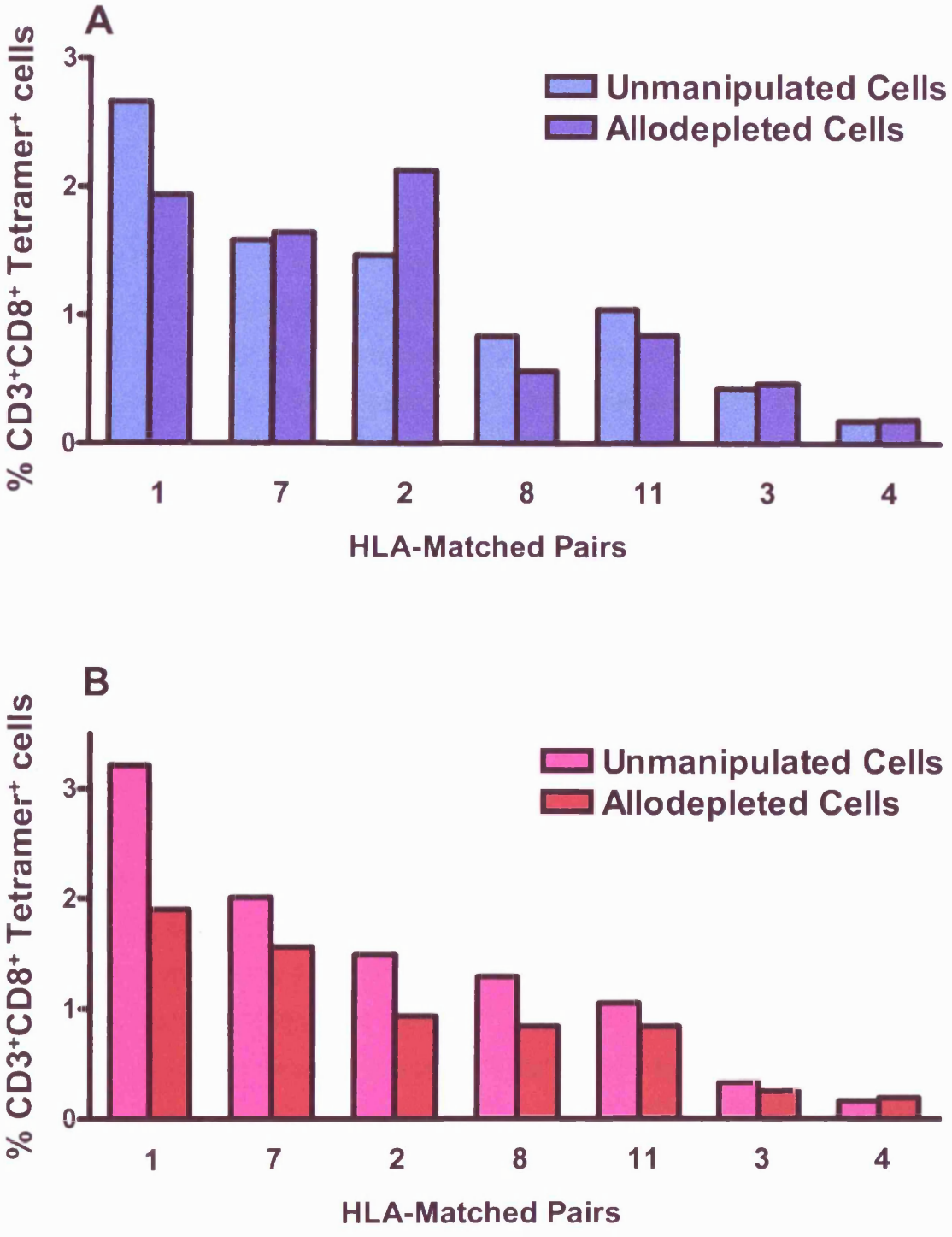

Figure 4.7 Retention of NLV-HLA A*0201 tetramer$^+$ cells after selective allodepletion in individual HLA-matched pairs. (Pairs are shown in descending order of frequency of NLV-HLA A*0201 tetramer$^+$ cells in unmanipulated cells);
(A). Cytokine allostimulation.
(B). OKT3 allostimulation.

4.4.8 Retention of CMV-peptide-stimulated IFN-γ ELISpot Reactive Cells in HLA-Matched Pairs

11 HLA-matched pairs (9 HLA A*0201$^+$) were studied for retention of CD3$^+$CD8$^+$ CMV-peptide-stimulated IFN-γ ELISpot-reactive cells The frequency of CD3$^+$CD8$^+$ CMV-peptide-stimulated IFN-γ ELISpot-reactive cells in responder cells after 72 hours of allostimulation was not significantly different to that of baseline, unmanipulated responder cells using either OKT3 pre-treated stimulators or cytokine pre-treated stimulators (data not shown).

After effective selective allodepletion of 11 HLA-matched pairs the frequency of CD3$^+$CD8$^+$ CMV-peptide-stimulated IFN-γ ELISpot reactive cells post-depletion was 76%+/-26% of the pre-depletion frequency utilising the OKT3 allostimulation technique and 89%+/-26% utilising the cytokine allostimulation technique (Figure 4.8). Post-depletion frequencies were not significantly different to pre-depletion frequencies in a paired, two-tailed Student's t-test ($p=0.28$) following the OKT3 allostimulation technique. The frequency of CD3$^+$CD8$^+$ CMV-peptide-stimulated IFN-γ ELISpot reactive cells post-depletion was higher after OKT3 allostimulation in the two pairs tested with the highest pre-depletion frequencies (Pairs 5 and 9) and lower in all the other pairs (Figure 4.9(A)). The retention of CD3$^+$CD8$^+$ CMV-peptide-stimulated IFN-γ ELISpot reactive cells post-depletion following the OKT3 allostimulation technique was not related to responder HLA type.

In a paired two-tailed Student's t-test the frequency of CMV ELISpot reactive cells in HLA-matched responders post-depletion was significantly lower than the value pre-depletion utilising the cytokine modified MLR ($p=0.05$), although this difference was not seen if HLA A*0201$^+$ responders alone were analysed ($n=9$, $p=0.21$). The frequency of CD3$^+$CD8$^+$ CMV-peptide-stimulated IFN-γ ELISpot reactive cells post-depletion was lower after cytokine allostimulation in 4 pairs tested (Pairs 5,9,10 and 1) and unchanged in all the other pairs (Figure 4.9(B)).

The retention of CD3$^+$CD8$^+$ CMV-peptide-stimulated IFN-γ ELISpot reactive cells after allodepletion following OKT3 allostimulation was not significantly different to that following cytokine allostimulation when all responders were analysed together ($n=11$, $p=0.2$ in an unpaired two-tailed Student's t-test), although there was a trend towards lower percentage retention after OKT3

allostimulation compared to cytokine allostimulation if HLA A*0201^{+} responders were analysed alone (n=9, p=0.07 in an unpaired two-tailed Student's t-test).

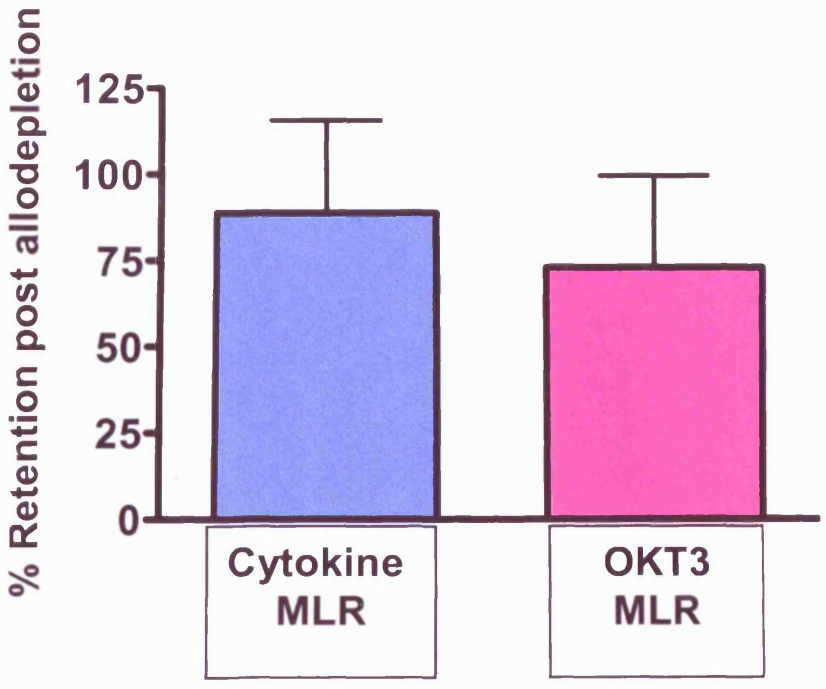

Figure 4.8 Retention of CMV-peptide-stimulated IFN-γ ELISpot-reactive cells after selective allodepletion in 11 HLA-matched pairs utilising the cytokine and the OKT3 allostimulation techniques. Error bars represent standard deviation. Percentage retention is frequency of $CD3^+CD8^+$ CMV-peptide-stimulated IFN-γ ELISpot reactive cells post-depletion x100/ frequency of $CD3^+CD8^+$ CMV-peptide-stimulated IFN-γ ELISpot reactive pre-depletion.

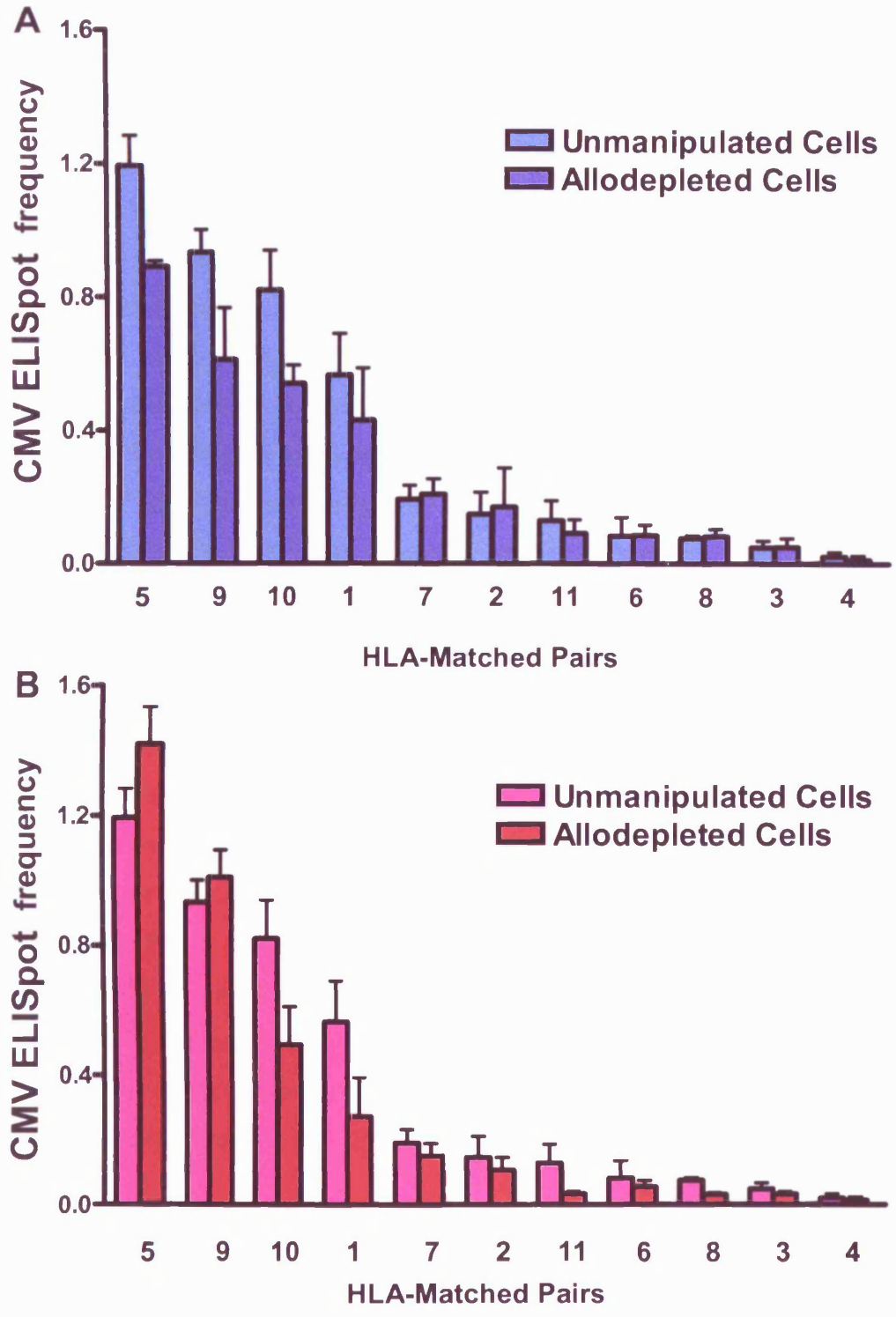

Figure 4. 9 Retention of CMV-peptide-stimulated IFN-γ ELISpot reactive cells after selective allodepletion in individual HLA-matched pairs. (Pairs are shown in descending order of frequency of $CD3^+CD8^+$ CMV-peptide-stimulated IFN-γ ELISpot reactive cells in unmanipulated cells).

(A). Cytokine allostimulation. (B) OKT3 allostimulation

4.4.9 Retention of EBV-peptide-stimulated IFN-γ ELISpot Reactive Cells in HLA-Matched Pairs

All matched pairs studied for retention of EBV-peptide-stimulated IFN-γ ELISpot reactive cells were HLA A*0201$^+$. The frequency of $CD3^+CD8^+$ EBV-peptide-stimulated IFN-γ ELISpot reactive cells in responder cells after 72 hours of allostimulation was not significantly different to that of baseline, unmanipulated responder cells using either OKT3 pre-treated stimulators or cytokine pre-treated stimulators (data not shown).

After effective selective allodepletion of 6 HLA-matched pairs the frequency of $CD3^+CD8^+$ EBV-peptide-stimulated IFN-γ ELISpot reactive cells post-depletion was 82%+/-16% of the pre-depletion frequency utilising the OKT3 allostimulation technique and 113%+/-23% utilising the cytokine allostimulation technique (Figure 4.10). Post-depletion frequencies were not significantly different to pre-depletion frequencies following the both the OKT3 and the cytokine allostimulation technique ($p=0.12$ and 0.75 respectively in a paired, two-tailed Student's t-test).

The frequency of $CD3^+CD8^+$ EBV-peptide-stimulated IFN-γ ELISpot reactive cells post-depletion was markedly lower than the pre-depletion frequency in only one of the 6 pairs tested (Pair 1) after OKT3 allostimulation and relatively unchanged in all other pairs (Figure 4.11A), whereas the frequency of $CD3^+CD8^+$ EBV-peptide-stimulated IFN-γ ELISpot reactive cells post-depletion was higher than the pre-depletion frequency in Pair 1 after cytokine allostimulation, lower in pair 2 and relatively unchanged in all other pairs (Figure 4.11(B)).

The retention of $CD3^+CD8^+$ EBV-peptide-stimulated IFN-γ ELISpot reactive cells after allodepletion following OKT3 allostimulation was significantly lower than that seen following cytokine allostimulation ($n=6$, $p=0.02$ in an unpaired two-tailed Student's t-test).

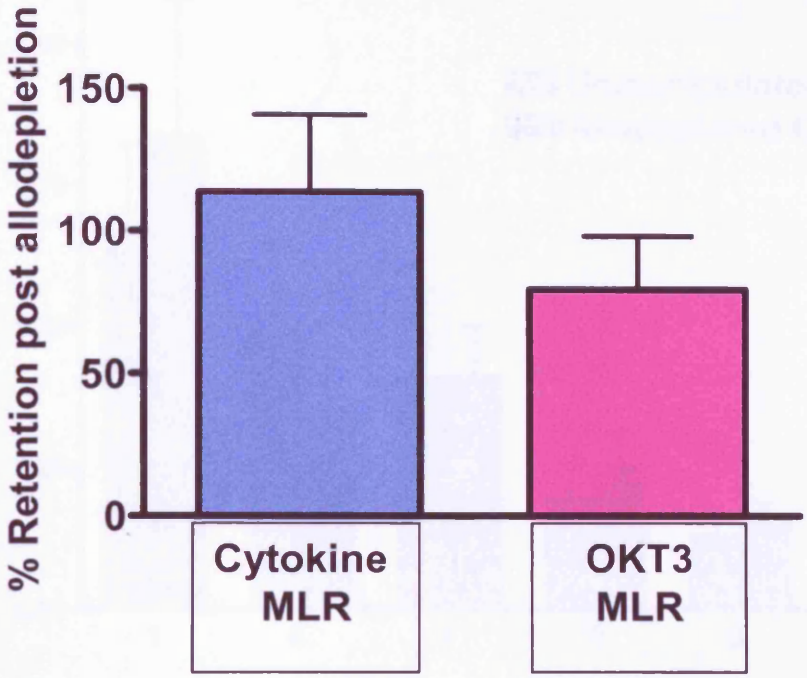

Figure 4.10 Retention of EBV-peptide-stimulated IFN-γ ELISpot reactive cells after selective allodepletion in 6 HLA-matched pairs utilising the cytokine and the OKT3 allostimulation techniques. Error bars represent standard deviation. Percentage retention is frequency of $CD3^+CD8^+$ EBV-peptide-stimulated IFN-γ ELISpot reactive cells post-depletion x100/ frequency of $CD3^+CD8^+$ EBV-peptide-stimulated IFN-γ ELISpot reactive pre-depletion.

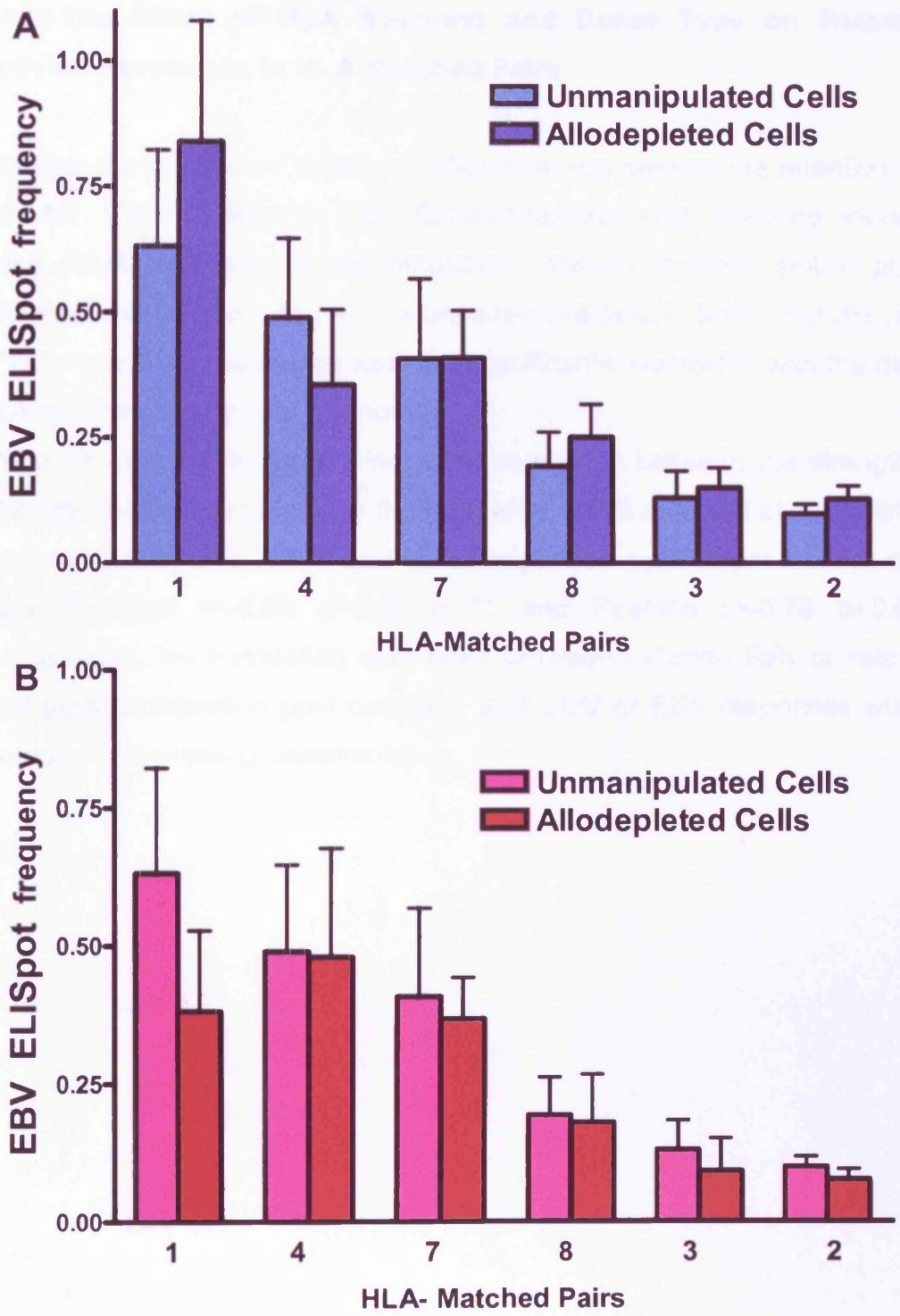

Figure 4.11 Retention of EBV-peptide-stimulated IFN-γ ELISpot-reactive cells after selective allodepletion in individual HLA-matched pairs. (Pairs are shown in descending order of frequency of ELISpot-reactive cells in unmanipulated cells);

(A). Cytokine allostimulation. (B). OKT3 allostimulation.

4.4.10 The Effect of HLA Matching and Donor Type on Retention of Antiviral Responses in HLA-Matched Pairs

Although numbers were small, no difference was seen in the retention of CMV tetramer, CMV ELISpot or EBV ELISpot-reactive cells following allodepletion using either technique of allostimulation between matched sibling pairs and matched unrelated donor pairs in unpaired two-tailed t-tests, and the retention of CMV and EBV responses were not significantly correlated with the degree of HLA matching in unrelated donor pairs.

There was a trend towards a negative correlation between the strength of the alloresponse (as measured by the RRI) after OKT3 modified allostimulation and the retention of both CMV and EBV responses by ELISpot in HLA-matched pairs (Pearson $r=-0.53$ $p=0.09$ $n=11$ and Pearson $r=-0.78$ $p=0.06$ $n=6$ respectively). No correlation was seen between cytokine RRI or retention of third party proliferation post depletion and CMV or EBV responses with either method of potentiating allostimulation.

4.5 Chapter Discussion

The preliminary experiments in fully HLA-mismatched pairs gave proof of principle that selective allodepletion based on the removal of $CD69^+$ responder cells after allostimulation retains the majority of CMV-specific cells in the allodepleted donor cell pool. The retention of CMV-specific CTLs identified by tetramer binding was demonstrated in HLA $A*0201^+$ responders and the retention of functional HLA A*0201-restricted CMV-specific CTLs after allodepletion was confirmed by IFN-γ ELISpot assay. In addition, retention of functional HLA B*0702-restricted CMV-specific CTLs after allodepletion was demonstrated by IFN-γ ELISpot assay.

Subsequent work in HLA A, B, C and DR matched sibling pairs and fully or partially matched unrelated donor pairs (using two different techniques to potentiate allostimulation) has confirmed retention of CMV-specific cells by tetramer (in HLA $A*0201^+$ individuals) and IFN-γ ELISpot (in HLA $A*0201^+$, $A*0101^+$ and $B*0702^+$ responders and retention of EBV-specific cells by IFN-γ ELISpot in HLA $A*0201^+$ responders. No prior data demonstrating preservation of specific cellular CMV and EBV responses following selective allodepletion based on CD69 expression in HLA-matched pairs and in non-HLA $A*0201^+$ individuals has been published.

Considerable variation between individuals was apparent in $CD3^+CD8^+$ NLV-HLA A*0201 $tetramer^+$ frequency. Baseline $CD3^+CD8^+$ NLV-HLA A*0201 $tetramer^+$ frequencies were 3-5-fold greater than baseline $CD3^+CD8^+$ NLV peptide-stimulated IFN-γ ELISpot reactive cells, which is in keeping with published literature. [Lechner *et al.* 2001] This difference may represent an excess of $tetramer^+$ cells that do not secrete IFN-γ, or simply differences in the sensitivity of the two assays.

In HLA-matched pairs no obvious pattern was seen in the degree of retention of any cellular response tested in relation to donor type or closeness of HLA matching but a small series such as this would only be able to detect such differences if they were very marked and independent of other variables.

In HLA-matched pairs both quantitative and qualitative differences in the retention of cellular antiviral responses after allodepletion were seen with the use of the two different techniques for allostimulation.

There was a trend for less retention of functional CMV-reactive cells (identified by ELISpot assay) and significantly less EBV retention in HLA A*0201$^+$ responders with the OKT3 technique than with the cytokine technique of allostimulation, despite very similar retention of third party proliferation post-depletion with the two techniques.

The negative correlation between the strength of the primary alloresponse (RRI, %) after OKT3 allostimulation and retention of CMV/EBV responses supports the concept of the existence of donor T cells with TCR affinity for both alloantigens and viral (e.g. EBV and/or CMV) antigens. OKT3 pre-treatment of stimulator cells may lead to sufficient upregulation of molecules involved in antigen presentation on the surface of stimulator T cells to enable weaker affinity interactions between MHC/antigen complexes and responder cell TCRs, which would favour the activation of responder TCRs with shared affinity for alloantigens and viral antigens.

Differences in the retention of viral responses within and between individuals in HLA-matched pairs seen with the two different allostimulation techniques may be a result of presentation of different lineage-specific allogeneic mHags (myeloid for the cytokine technique and lymphoid for the OKT3 technique) to responder T cells, with different degrees of TCR shared affinity for viral antigen.

Nevertheless the demonstration of retention of the majority of functional CD8$^+$ cellular CMV- and EBV-specific responses after efficient allodepletion of HLA-matched pairs has great significance for the development of the CD69 allodepletion strategy to a clinical scale.

Further studies need to focus on retention of CD4$^+$ antiviral responses as well as CD8$^+$ responses. It must be stressed that the viral peptide-stimulated IFN-γ ELISpot assay measures only CD8$^+$ CTL responses, and gives no information about HLA Class II-restricted CD4$^+$ effector and memory cells that are known to be required for the persistence of cellular immunity to CMV.[Gamadia *et al* 2003;Zajac *et al* 1998] Experiments using professional antigen presentation with dendritic cells and whole CMV protein would be needed to examine the preservation of CD4$^+$ cellular anti-CMV and –EBV activity after CD69-based selective allodepletion.

The benefit of preservation of CMV CD8$^+$ responses in selectively allodepleted cell pools would clearly be to confer adoptive anti-CMV immunity to the recipient of a selectively allodepleted AHSCT, as CMV infection is still a common

occurrence post-transplant although prophylactic antiviral therapy and monitoring with real-time PCR for CMV DNA and pre-emptive treatment has reduced the incidence of clinically severe CMV disease and mortality directly attributable to the disease.

The importance of retaining EBV-specific cellular responses after selective allodepletion based on CD69 expression is in the potential for adoptive transfer of such cells within selectively allodepleted grafts to recipients and the subsequent protection against EBV-related PTLD.

Whilst the majority of the EBV-related PTLD lymphoid expansions have been associated with latent type EBV infection, the EBV lytic cycle also appears to play an important role in PTLD. PTLD tumours have been examined with specific antibodies for detection of early and late lytic-cycle antigens, or with oligoprobes to measure the presence of latent- and lytic-cycle viral DNA. Under immunosuppressive therapy up to 80% of tumours examined showed evidence of EBV early lytic cycle reactivation and 30-40% of the tumours contained EBV late lytic antigens or replicative DNA. Strong evidence has also been gathered from animal models showing that the lytic cycle may be important in the development of PTLD. SCID mice injected with EBV-immortalized B-cell populations in the latent or lytic state revealed that mice injected with the lytic-positive population developed tumours more readily.[Tanner and Alfieri 2001] Thus the demonstration of retention of EBV lytic peptide GLC-specific CTL responses can be taken as evidence that CD69 allodepleted donor cell fractions could confer protection against EBV-related PTLD, (although EBV-specific responses to other latent-cycle peptides clearly also need to be studied).

NLV-CMV and GLC-EBV-specific T cell are frequent in most HLA A*0201$^+$ individuals although T cells responding to both peptides are often less frequent than T cells responding to B*0702- restricted peptides in A*0201$^+$ B*0702$^+$ individuals.[Hollsberg 2002;Kern *et al.* 1999] In such individuals it would be prudent to measure CMV and EBV responses to both HLA A*0201$^+$ and B*0702$^+$-restricted CMV and EBV-peptides.

Viruses other than CMV and EBV are controlled primarily by a T cell mediated immune response and are important pathogens post AHSCT. These include respiratory syncytial virus, and adenovirus.[Alwan *et al.* 1992;Walls *et al.* 2003] Life-threatening complications arising from infection from these viruses post-AHSCT have been treated successfully with donor lymphocyte infusions, with

presumed transfer of antiviral CTLs.[Hromas *et al.* 1994;Kishi *et al.* 2000] Specific cellular antiviral immunity to such viruses might also be preserved by selective alloreactive T cell depletion of the donor T cell pool.

Chapter 5 Phenotypic and Functional Characteristics of Alloreactive and Non-alloreactive Cells

5.1 Introduction

In order to provide effective quantitative and qualitative broad spectrum T cell reconstitution post-AHSCT, the graft T cell pool should contain antigen specific $CD4^+$ and $CD8^+$ T cells of both memory and effector phenotype.

Memory T cells can be differentiated from naïve T cells by their relative expression of isoforms of the TCR stabilising molecule CD45, a transmembrane tyrosine phosphatase. The CD45 isoform expressed on the T cell surface changes after prolonged antigenic stimulation. Alternate splicing of exons that encode the extracellular domain of CD45, leads to increased production of CD45RO, (the isoform that binds and stabilises the TCR and facilitates antigen recognition) and decreased production of the high molecular weight CD45RA isoform that does not associate with the TCR or with the CD4 or CD8 molecules. Memory and naïve T cells can thus be broadly identified by expression of CD45 isoform.[Janeway *et al.* 1999]

Lanzavecchia and Sallusto have further classified both $CD4^+$ and $CD8^+$ $CD45RO^+CD45RA^-$ memory T cells based on functional characteristics and expression of the chemokine receptor CCR7.[Lanzavecchia and Sallusto 2000;Sallusto *et al.* 1999] Central Memory and Effector Memory T cells (T_{CM} and T_{EM}) were initially defined in the human system based on two distinct criteria;

(a). the expression of homing receptors such as CCR7 that allow cells to migrate to secondary lymphoid organs versus non-lymphoid tissues;
(b). the absence or presence of immediate effector function.

CCR7 is a transmembrane G-protein-coupled receptor important in lymphocyte trafficking and required for diapadesis through high endothelial venules (HEV) and migration to T cell areas of secondary lymphoid organs. [Campbell *et al.* 1998;Forster *et al.* 1999] Human T_{CM} are $CD45RO^+$ memory cells that constitutively express CCR7. When compared with naïve T cells, T_{CM} have higher sensitivity to antigenic stimulation, and are less dependent on co-

stimulation. They also upregulate CD40 ligand effectively and are therefore able to provide effective stimulatory feedback to dendritic cells and B cells. Following TCR triggering, T_{CM} produce mainly IL-2, but after proliferation they efficiently differentiate to effector cells and produce large amounts of IFN-γ or IL-4. Human T_{EM} are memory cells that have lost their constitutive expression of CCR7 and display characteristic sets of chemokine receptors and adhesion molecules that are required for homing to inflamed tissues. When compared with T_{CM}, T_{EM} are characterized by rapid effector function. $CD8^+$ T_{EM} carry large amounts of perforin, and both $CD4^+$ and $CD8^+$ cells produce IFN-γ, IL-4, and IL-5 rapidly following antigenic stimulation. The T_{EM} pool contains $CD4^+$ cells able to mediate both T_H1 and T_H2 type responses, and $CD8^+$ CTLs.[Sallusto *et al* 1999] [Campbell *et al.* 2001]Other work has tried to classify memory T cell subsets based on expression of co-stimulatory molecules such as CD27 and CD28. These molecules, which are expressed on naïve T cells, are also expressed on some memory T cells, however they are absent in a subset of $CD8^+$ memory T cells characterized by high effector function and expression of CD45RA.[Appay *et al.* 2002;Hamann *et al.* 1997] The combination of CCR7 and CD45RA expression can thus divide $CD4^+$ T cells into 3 functional subsets and $CD8^+$ T cells into 4 functional subsets, although considerable heterogeneity of expression of other chemokine receptors, adhesion molecules and co-stimulatory molecules exists within these subsets (Figure 5.1).[Sallusto *et al.* 2004a]. Expression of CD62L (L selectin), which also guides lymphocytes into lymphoid tissue, is closely linked to CCR7 expression on memory $CD4^+$ T cells and thus T_{CM} and T_{EM} cells may also be differentiated based on their expression of CD62L.[Sallusto *et al* 1999]

Proliferative responses in HLA-mismatched MLRs have been reported with purified ($CD4^+$ and $CD8^+$) $CD45RA^+$ and $CD45RO^+$ responder cells indicating functional alloreactive cells are contained in both naïve and memory cell subsets. [O'Brien and Kemeny 1998]

Recently an increase in the T_{EM} phenotype ($CCR7^-$ $CD62L^{low}$) of circulating T cells has been reported in AHSCT recipients with chronic GVHD, although the distribution between central and effector memory cell subsets in alloreactive cells has not been yet been described.[Yamashita *et al.* 2004]

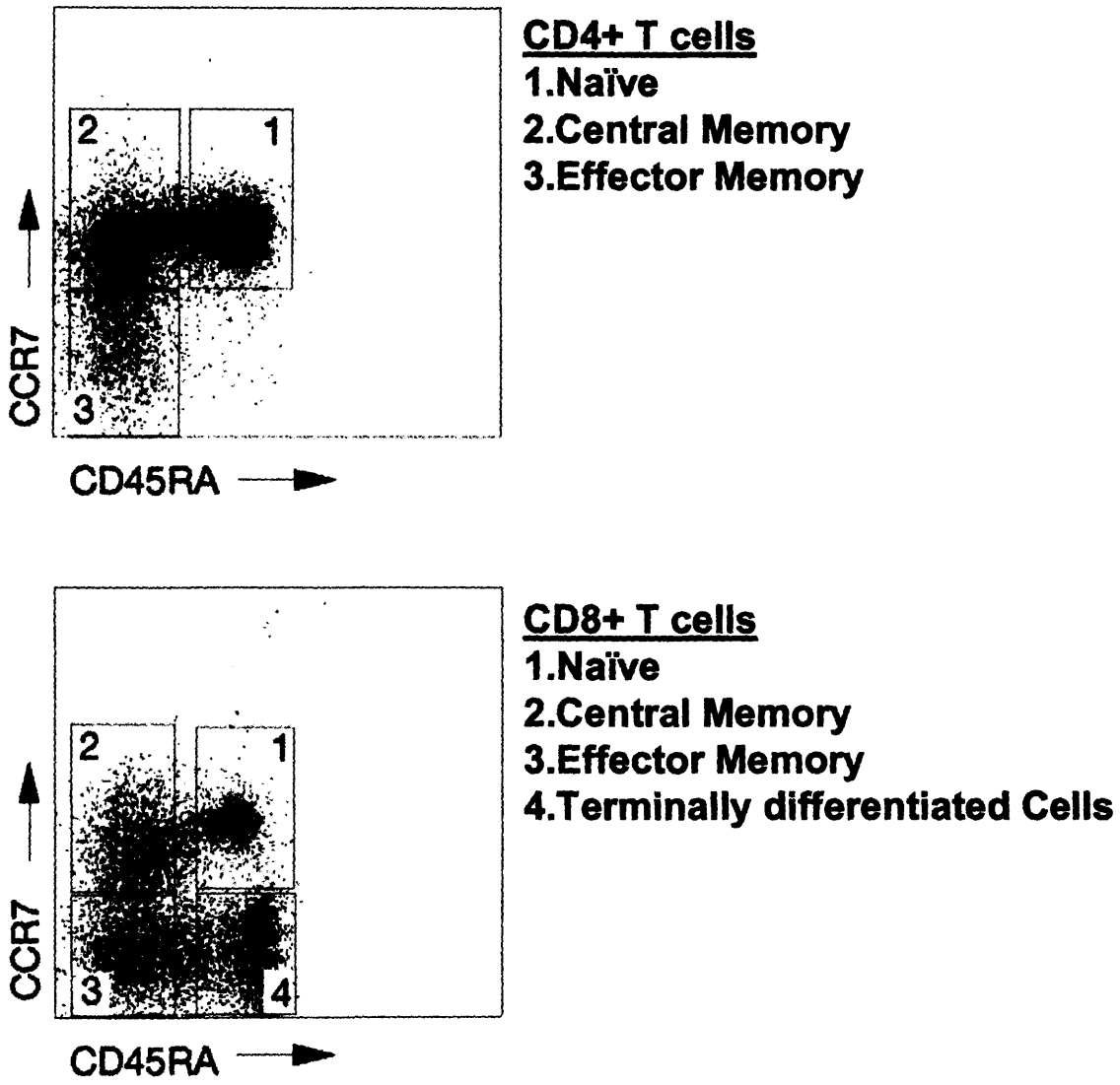

Figure 5.1 The Classification of $CD4^+$ and $CD8^+$ T cells into Naïve, Central and Effector Memory Cells based on expression of the chemokine receptor CCR7 and CD45RA. Figure taken from Lanzavecchia and Sallusto.[Lanzavecchia and Sallusto 2000;Sallusto *et al* 1999]

The TCR is a molecular complex comprising two units; a variable 'recognising unit' (either the αβ heterodimer or in 1-10% of T cells the γδ heterodimer) and a transducing unit that allows a signal to be delivered to the T cell. The 'recognising unit' requires great variability in order to bind different antigen/MHC molecules and this is achieved by the somatic genomic recombination process in the thymus. Each of 4 TCR gene loci (α, β, γ and δ) is composed of several highly polymorphic V, D and J segments and one or two constant genes. During T cell gene rearrangement in any given T cell, random V, D, J and C genes are joined together to form a single and unique combination. There are 65 Vβ segments in the β locus that can be grouped into 25 sub-families (22 functional sub-families).[Neisig *et al.* 1993;Peggs *et al.* 2003b] The measurement of the relative frequencies of $CD4^+$ and $CD8^+$ cells within each of these Vβ sub-families is able to give a measure of the diversity of T cells within a given cell pool. Oligoclonal or clonal restriction of T cells within a given TCR Vβ sub-family can be determined by the use of a set of PCR primers specific to each TCR Vβ sub-family, to amplify the 3rd complimentarity determining region (CDR3) length of DNA. A distribution histogram (the spectratype) can be produced for each TCR Vβ sub-family.[Gorski *et al.* 1994] Polyclonal responses are characterised by the conservation of baseline CDR3 length distributions within each TCR Vβ sub-family with multiple peaks in the spectratype, and normal spectratypes for all TCR Vβ sub-families. Restricted clonal responses demonstrate a single sharp peak with absence of the other peaks within a given TCR Vβ sub-family. Although CDR3 PCR is only semi-quantitative, in highly restricted clonal T cell responses all T cells may belong to the same TCR Vβ sub-family and the normal pattern within other TCR Vβ sub-families is absent, and flat spectratypes are seen. Antibodies are now available to epitopes for most common TCR Vβ sub-families that permit the direct assessment of TCR Vβ sub-family distribution of individual cell subsets. Although this technique is quantitative, it has a lower resolution when compared to CDR3 PCR and no information about distribution of TCR Vβ rearrangements within a given sub-family can be derived. Additionally there is no consensus about how the degree of diversity of TCR Vβ sub-family distribution should be expressed following direct quantification by flow cytometric techniques, whereas several mathematical models have been constructed to describe diversity in CDR3 length frequencies both within and

between TCR Vβ sub-families.[Bomberger *et al.* 1998;Dumont-Girard *et al.* 1998;Godthelp *et al.* 1999;Gorochov *et al.* 1998]

Human T cell responses to certain pathogens (e.g. Influenza A) are characterised by the presence of cells with a highly restricted TCR Vβ sub-family usage, which may be constant between different individuals.[Lehner *et al.* 1995] Other pathogens (e.g. EBV, CMV) are characterised by less focussed T cell responses, with pathogen-specific T cells demonstrating restriction to several Vβ sub-families within individuals, even in cell populations specific to an identified peptide antigen and selected cell sorting based on expression of MHC Class I-peptide tetrameric complexes.[Lim *et al.* 2000b;Peggs *et al.* 2002]

Alloreactive cells were first shown to have restricted TCR Vβ sub-family use when DNA was analysed by PCR for CDR3 length in alloreactive cell clones specific for identified MHC Class I and II molecules.[Li *et al.* 1996;Lobashevsky *et al.* 1996] TCR Vβ sub-family usage has also been shown to be restricted in T cell lines derived from cells infiltrating renal allografts.[Hall and Finn 1992]

Recently anergic T cells capable of suppressing both the cytokine and proliferative responses of other T cells have been identified and these *T regulatory cells* are present in healthy individuals. [Ng *et al.* 2001]

Although not identified by a highly specific cell surface antigen and not exclusive to a readily identifiable cell subset, they are enriched within the subset of resting T cells that co-express the CD4 receptor and the low affinity IL2 receptor CD25.

These $CD4^+CD25^+$ T regulatory cells form less than 10% of resting human $CD4^+$ lymphocytes, but are increasingly recognised as playing an important role in suppression of immune responses both in healthy human subjects and in animal models of allotransplantation.[Papiernik 2001] $CD4^+CD25^+$ T regulatory cells are required for the *ex vivo* induction of tolerance to alloantigen via co-stimulatory blockade and to inhibit allogeneic skin graft rejection.[Field and Gao 1998;Gao *et al.* 1999;Jarvinen *et al.* 2003] There is also evidence that these cells can suppress the alloreactive response and limit the development of allograft rejection. [Kingsley *et al.* 2002;van Maurik *et al.* 2002;Zhang *et al.* 2001] Such T regulatory cells have been shown to suppress GvHD responses in mice. Depletion of $CD4^+CD25^+$ T regulatory cells from donor T cell inoculum or *in vivo* CD25-depletion of the recipient before transplantation resulted in

increased GvHD mediated by $CD4^+$ cells or unselected T cells in mice. The infusion of freshly purified donor $CD4^+CD25^+$ T regulatory cells modestly inhibited GvHD when administered in equal numbers with $CD4^+$ cells but at higher levels significantly inhibited rapidly lethal GvHD.[Taylor *et al* 2002]
CD25 is an important T cell activation antigen and as such has been used to identify and selectively remove alloreactive cells by several groups. Such a strategy is likely to remove $CD4^+CD25^+$ T regulatory cells although the impact of this is not known. No data have been published on the co-expression of CD69 and CD25 on alloreactive cells following allostimulation. A potential difference between the CD69-mediated allodepletion strategy and those based on CD25 expression would be the retention of $CD4^+CD25^+$ T regulatory cells.

5.2 Aims of the Experiments Described in this Chapter

1. To determine the memory/effector phenotype of both alloreactive and non-alloreactive cells;
2. To determine the TCR Vβ sub-family distribution in both alloreactive and non-alloreactive cell subsets in both the HLA-mismatched and matched setting;
3. To determine the co-expression (if any) of the activation antigens CD69 and CD25 on cells after allostimulation and timescale of expression of these antigens in HLA-mismatched and HLA-matched settings;
4. To determine whether functional $CD4^+CD25^+$ T regulatory cells are retained in the non-alloreactive cell fraction following selective alloreactive cell depletion by immunomagnetic sorting of $CD69^+$ cells.

5.3 Methods and Experimental Design

5.3.1 Memory and Effector Phenotype of Alloreactive cells

20 healthy subjects, (11 male and 9 female) were studied to generate normal values for $CD4^+$ T_{CM}, T_{EM}, and naïve cells and $CD8^+$ T_{CM}, T_{EM}, naïve and terminally differentiated cells. Four-colour flow cytometry was performed on whole blood using the following antibody panels:

Fluorochrome	Panel 1	Panel 2
FITC	CCR7/GAM	CD56
PE	CD4	CD20
PerCP	CD8	CD8
APC	CD45RA	CD3

Table 5.1 Antibody panels used for determination of naïve and memory T cell subsets in healthy controls.

Antibodies used were all from Becton Dickinson and the antibody staining procedure was as described in Chapter 2.2.1. Panel 1 was used to determine the percentage of $CD4^+$ and $CD8^+$ cells within the live lymphocyte region on a FSC vs. SSC dot plot within each quadrant of a CD45RA vs. CCR7-GAM dot plot. Panel 2 was used to determine the percentage of $CD3^-CD56^+$ NK cells, $CD3^+$ T cells, $CD3^+$ $CD56^+$ NK-T cells, $CD20^+$ B cells, $CD3^+$ $CD8^+$ T cells and $CD3^+$ $CD56^-$ $CD8^-$ T cells ($CD4^+$ cells) within the live lymphocyte gate. The total lymphocyte count was quantified on the same whole blood sample on an Advia automated cell counter. The analysis of the data from panels 1 and 2 and the total lymphocyte count allowed the calculation of absolute values for naïve and memory cell subsets for each individual.

6 different HLA-mismatched MLRs were set up and cells were phenotyped after 72 hours of co-culture with the panels described below using the phenotyping protocol described in Chapter 2.2.1

Fluorochrome	Panel 1	Panel 2
FITC	CCR7/GAM	CCR7/GAM
PE	CD69	CD69
PerCP	CD4	CD8
APC	CD45RA	CD45RA

Table 5.2 Antibody panels used for determination of naïve and memory T cell subsets in responder cells in HLA-mismatched MLRs.

5.3.2 TCR Vβ Sub-family Phenotyping

TCR Vβ sub-family distribution was assessed by flow cytometry using the IOTest Beta Mark kit (Beckman-Coulter, USA). Briefly this kit comprises 24 murine and rat monoclonal and polyclonal antibodies directed against epitopes of 24 of the commonest human TCR Vβ sub-families, covering 67% of TCR Vβ sub-families expressed on human $CD8^+$ T cells and 72% on $CD4^+$ T cells. 8 mixtures of 3 antibodies (murine IgM and IgG (22 antibodies) and rat IgM and IgG (one each) are supplied, each mixture containing a PE-conjugated antibody with specificity against one TCR Vβ sub-family, a FITC-conjugated antibody with specificity against a second, and a third antibody with specificity against a third TCR Vβ sub-family which is conjugated to both PE and FITC fluorochromes. 10µl of each antibody mixture was added to 0.5-1x10^6 PBMCs along with 5µL of CD4/8 PerCP and 5µl CD69 APC (both BD) and incubated at room temperature in the dark for 15 minutes. (No CD3 antibody was used as preliminary experiments demonstrated that anti-CD3 antibody binding interfered with anti-TCR Vβ antibody binding). Cells were washed once in PBS for 10 minutes and resuspended in 300µL PBS. The cells were acquired within 4 hours of staining on a FACSCalibur flow cytometer (Becton Dickinson) in four-colour mode.

Events were gated on a region (R1) drawn around the live lymphocyte region of normal controls or responder cells from MLRs on a FSC vs. SSC dot plot. For experiments where TCR Vβ sub-family distribution was assessed in $CD4^+$ and

$CD8^+$ subsets, further regions were drawn around $CD8^{bright}$ (or $CD4^+$) cells (R2) or $CD8^-$ (or $CD4^-$) cells (R3) on a FSC vs. CD4/8 Per CP histogram gated on R1 (G1). A further CD69-APC histogram was generated, gated on (R1+R2) (G2) or (R1+R3), (G3). Regions were drawn around the $CD69^-$ cells (R4) and the $CD69^+$ cells (R5). Finally TCR Vβ FITC vs. TCR Vβ PE dot plots were generated gated on (G2+R4), (G3+R4), (G2+R5) and (G2+R6). The relative percentage of cells expressing each TCR Vβ was determined from cluster analysis of these dot plots. This gating strategy is illustrated in Figure 5.1.

The normal ranges were derived in this way from 12 healthy adult individuals (7 male, 5 female) using this staining protocol (with CD8 PerCP and without CD69 antibody) to validate the TCR Vβ antibody staining on PBMCs.

The flow cytometric measurement of TCR Vβ sub-family distribution was chosen as it enabled discrimination of CTLs responding to stimuli (based on CD69expression) without more complex cell sorting procedures. The Beta Mark kit only allows the assessment of the percentage of CTLs in each TCR Vβ sub-family (and does not therefore give the level of resolution of TCR spectratyping by PCR for CDR3 length). However DNA spectratyping cannot distinguish between DNA from different cell populations and would not be therefore be suitable for TCR assessment of responder cells in the allogeneic MLR as these cells are contaminated with irradiated stimulator cells. Moreover molecular techniques do not allow the assessment of TCR Vβ sub-family repertoire on T cell subsets and on cells expressing activation markers (e.g. CD69) without first using cumbersome cell sorting procedures.

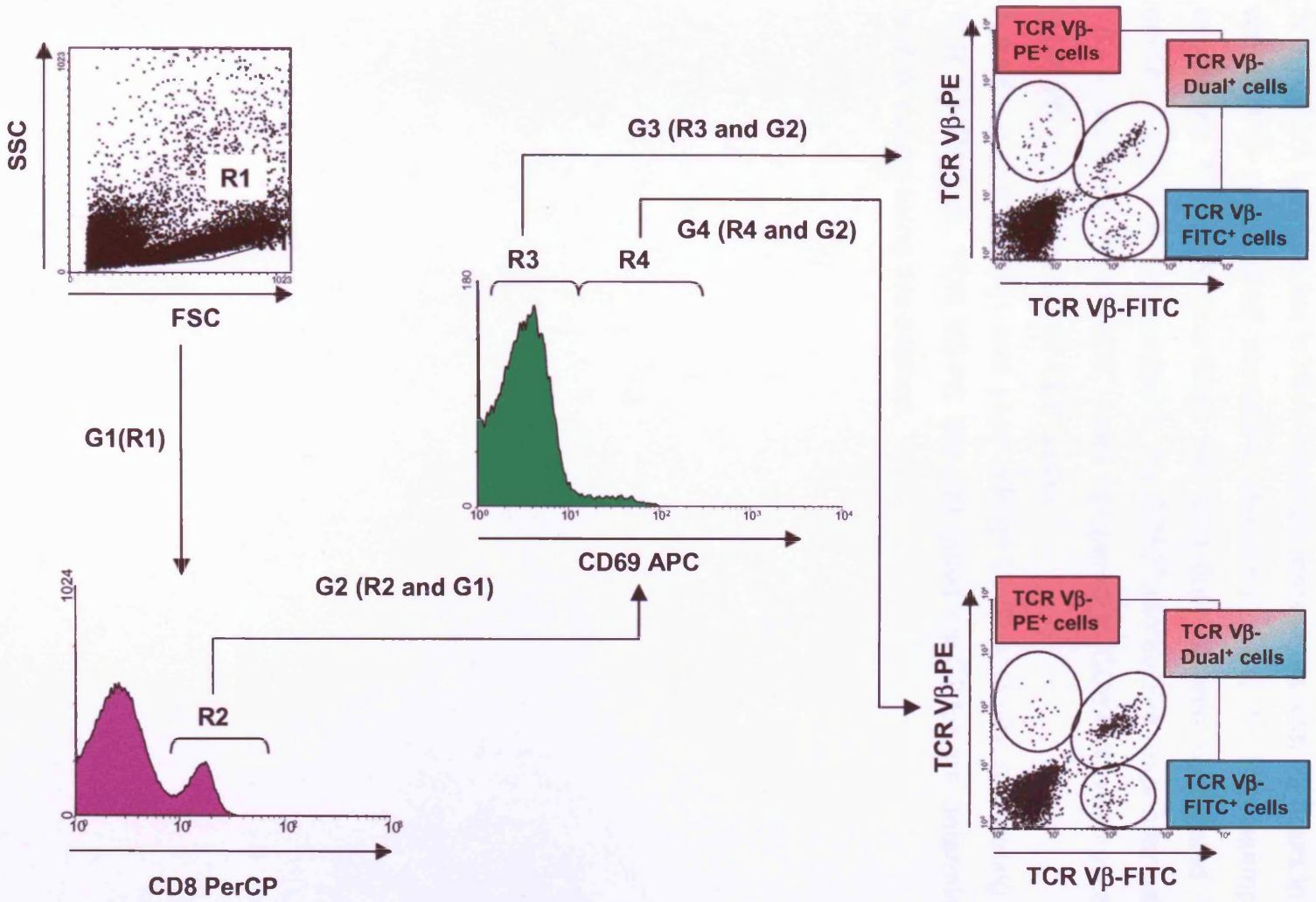

Figure 5.2 (See over for figure legend).

Figure 5.2 Gating strategy for flow cytometric determination of TCR Vβ sub-family distribution of responders in MLR. G1=R1, G2=(R1 and R2), G3=(G2 and R3), and G4=(G2 and R4). This example demonstrates the relative frequencies of 3 TCR Vβ sub-families in HLA–matched responders after 72 hours in an MLR with OKT3 pre-treated irradiated stimulator cells. In this example cells expressing TCRVβ-1 (identified the dual fluorochrome conjugated TCR Vβ antibody) are over-represented in the $CD69^+$ alloreactive responder cells (TCR Vβ -1 frequency 8% of $CD8^+$ cells) compared to $CD69^-$ non-alloreactive cells (TCR Vβ-1 frequency 3% of $CD8^+$ cells).

A similar gating strategy was used without CD69 antibody, generating a single TCR Vβ-FITC vs. TCR Vβ-PE dot plot gated on G2.for the determination of normal values using this method.

5.3.3 CD69 and CD25 Co-expression on Alloreactive Cells

HLA-matched and HLA-mismatched MLRs were set up as described in Chapter 2.3.2-4.

Responder cells were stained with the following antibody panel after 48, 72, 96 and 120 hours of incubation in the MLR. All antibodies were supplied by BD and the staining protocol was as detailed in Chapter 2.2.1.

Fluorochrome	Antibody
FITC	CD3
PE	CD25
PerCP	CD8
APC	CD69

Table 5.3 Antibodies used for flow cytometric assessment of the co-expression of CD69 and CD25 on responder cells in MLRs.

5.3.4 $CD4^+CD25^+$ T regulatory Cells in the MLR

The frequency of $CD4^+CD25^+$ T regulatory cells was assessed before and after allostimulation and after selective depletion of $CD69^+$ alloreactive cells (by immunomagnetic sorting with Miltenyi system, Chapter 2.5.1) in HLA-mismatched and HLA-matched responders using the antibody panel shown in Table 5.3.

$CD4^+CD25^+$ T regulatory cells in selectively allodepleted HLA-mismatched responder cells were stained with CD25-PE and CD4-PerCP (both BD) and sorted on a FACSVantage SE high speed cell sorter (BD) to enrich the $CD4^+CD25^+$ T regulatory cell fraction to a purity of >80%. These sorted $CD4^+CD25^+$ T regulatory cells were added at a ratio of 1:1 to an HLA-mismatched third party MLR (with responders autologous to the $CD4^+CD25^+$ T regulatory cells) in order to assess the immunosuppressive capacity of the $CD4^+CD25^+$ T regulatory cells after selective allodepletion. The non-$CD4^+CD25^+$ T regulatory cell fraction from the sort was also added to the HLA-mismatched

third party MLR (with responders autologous to the non-$CD4^+CD25^+$ T regulatory cell fraction) as a negative control. The experimental schema is illustrated in Figure 5.3 (overleaf).

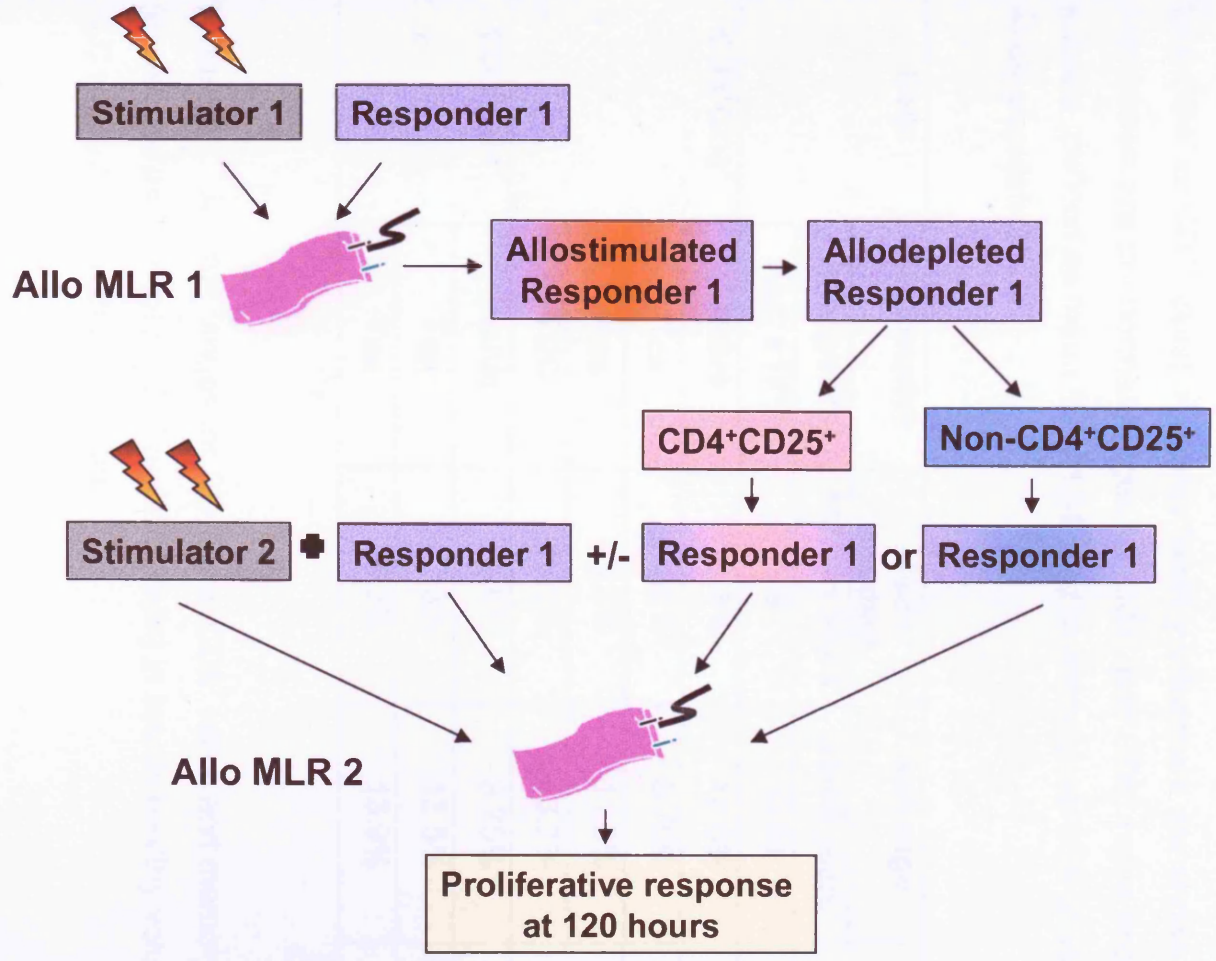

Figure 5.3 Experimental schema for functional assessment of $CD4^+CD25^+$ T regulatory cells after selective allodepletion in HLA-mismatched MLRs.

5.4 Results

5.4.1 Naïve and Memory T Cell Subsets in Normal Controls

The age, absolute lymphocyte count, and mean and standard deviation for $CD4^+$ and $CD8^+$ naive and memory cell subsets (expressed as percentage of total $CD4^+$ or $CD8^+$ cells) in twenty healthy volunteers are shown in Table 5.4. Also shown are the normal ranges for $CD4^+$ and $CD8^+$ naive and memory cell subsets (defined as mean % +/-1.96 x sd to encompass 95% of values from the whole population).

Cells	Parameter	Mean /median	sd/range	Normal Range
	Age (years)	Median 36yrs	25-65 years	
	ALC $x10^9$/L	1.9	0.54	0.84-2.9
$CD3^+CD8^+$	**Naïve**	21.3%	10.6%	0.52-42%
	T_{CM}	9.60%	6.70%	0-23%
	T_{EM}	45.6%	11.5%	23-68%
	TDC	23.5%	13.7%	0-50%
$CD3^+CD4^+$	**Naïve**	24.6%	8.70%	7.2-42%
	T_{CM}	37.3%	13.5%	10-64%
	T_{EM}	36.2%	13.9%	8.4-64%

Table 5.4 Normal ranges for $CD4^+$ and $CD8^+$ naive and memory cell subsets (expressed as % of total $CD4^+$ or $CD8^+$ cells) in twenty healthy volunteers.
ALC = absolute lymphocyte count.

5.4.2 Naive and Memory Subsets in CD69$^+$Alloreactive Cells

The percentage CD4$^+$ and CD8$^+$ naive and memory cell subsets in baseline and CD69$^+$ alloreactive cells in 6 HLA-mismatched responder cells after 72 hours of allostimulation in a standard MLR are shown in Figure 5.4.

CD69$^+$ alloreactive HLA-mismatched responders contain a mixture of naïve, memory and terminally differentiated CD8$^+$ cells. Although not reaching statistical significance there was a trend towards a higher frequency of CD8$^+$ T_{EM} in CD69$^+$ cells than in baseline responder cells ($p=0.07$ in a paired two-tailed t-test). In 4 of the 6 HLA-mismatched pairs studied the frequency of CD8$^+$ naïve cells in CD69$^-$ non-alloreactive cells after 72 hours of allostimulation was above the normal range seen in baseline healthy CD8$^+$ cells.

CD69$^+$ alloreactive HLA-mismatched responders contained a mixture of both naïve and memory CD4$^+$ cells, although a significantly lower frequency of CD4$^+$ naïve cells and a significantly higher frequency of CD4$^+$ T_{EM} cells was seen in CD4$^+$CD69$^+$ alloreactive cells compared to baseline responder cells ($p=0.049$ in a paired two-tailed Student's t-test). An increase in the frequency of total CD4$^+$ memory cells (T_{CM} +T_{EM}) was seen in 5 of the 6 mismatched pairs.

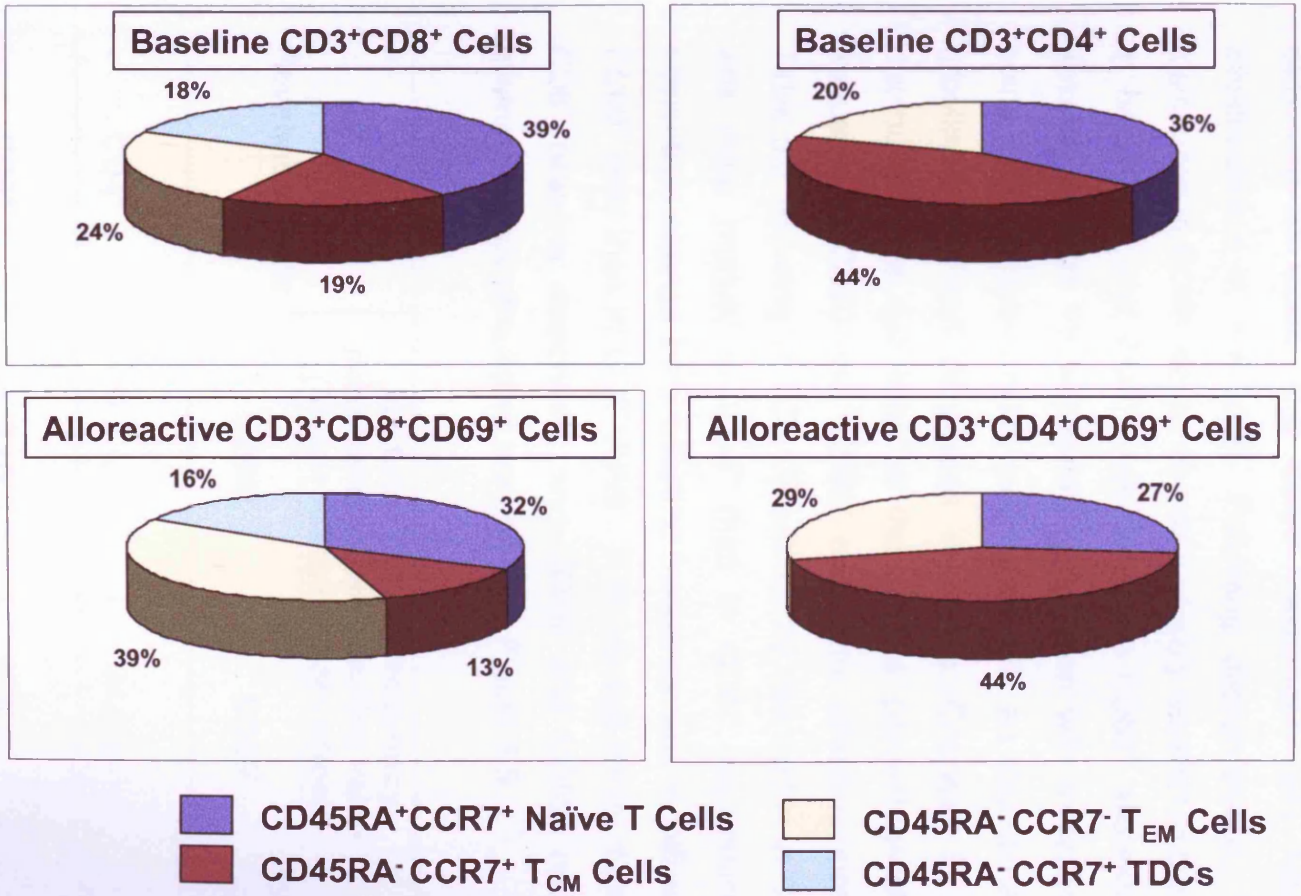

Figure 5.4 Memory and effector phenotype of baseline and alloreactive $CD3^+CD8^+$ and $CD3^+CD4^+$ cells. T_{CM} = Central Memory cells, T_{EM} = Effector Memory cells, TDC = Terminally Differentiated cells based on expression of CD28 and CCR7, as described by Lanzavecchia and Sallusto[Lanzavecchia and Sallusto 2000]

5.4.3 TCR Vβ Sub-family Frequency Distribution in Cells after Allostimulation

Responder TCR Vβ sub-family distribution was determined in 4 HLA-mismatched stimulator-responder pairs in baseline (unstimulated) responder cells and in $CD69^-$ and $CD69^+$ responder cells following 72 hours of allostimulation in the MLR. Following allostimulation, TCR Vβ sub-family distribution in $CD69^-$ responder cells closely resembled the baseline distribution in both $CD4^+$ and $CD8^+$ cells, whereas $CD69^+$ alloresponder cells showed skewing of TCR Vβ sub-family distribution with increases in frequencies of some TCR Vβ sub-families (consistent with an oligoclonal T cell response to allostimulation) and decreases in others. Changes in TCR Vβ sub-family distribution were quantified as the modular percentage change from baseline frequency in $CD69^-$ and $CD69^+$ cells after allostimulation and are shown in Table 5.6. Skewing of TCR Vβ sub-family distribution in $CD69^+$ alloresponders was more marked in $CD8^+$ than in $CD4^+$ responders and the modular percentage change from baseline frequency was significantly greater in $CD8^+$ $CD69^+$ cells than in $CD8^+CD69^-$. TCR Vβ sub-family distribution in $CD4^+$ and $CD8^+$ baseline responders and $CD69^-$ and $CD69^+$ responders after HLA-mismatched allostimulation are shown in Figure 5.5.

Responder Cells	**Modular TCR Vβ frequency pertubation from baseline value. (Mean+/-standard deviation).**		**P value in paired two-tailed Student's T-test**
	$CD69^-$	**$CD69^+$**	
$CD4^+$	*19.3 +/- 0.6*	*31.5 +/-7.8*	*0.10*
$CD8^+$	*26.3 +/- 5.5*	*56.9 +/- 12.9*	*0.03*

Table 5.5 Pertubation from baseline frequencies of TCR Vβ sub-families in $CD69^-$ and $CD69^+$ responder cells after HLA-mismatched allostimulation.

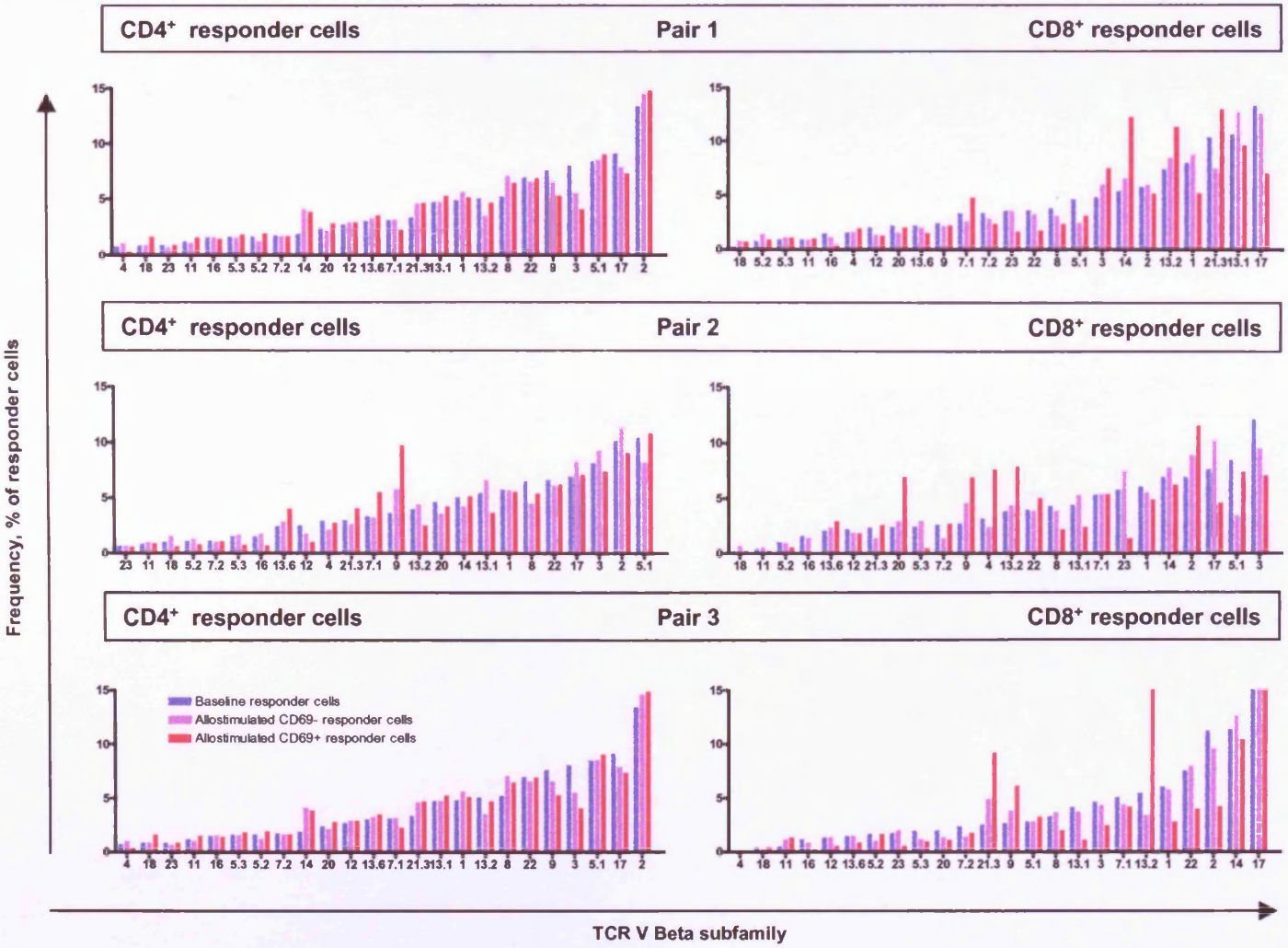

Figure 5.5 See over for Figure Legend

Figure 5.5 TCR Vβ sub-family distributions in baseline, $CD69^-$ and $CD69^+$ responder cells after 72 hours in the MLR with HLA-mismatched stimulator cells. Results are shown for $CD4^+$ and $CD8^+$ cells in 3 stimulator-responder pairs. TCR Vβ sub-families are plotted in ascending frequency in baseline responder cells for each stimulator responder pair.

Responder TCR Vβ sub-family distribution was also determined in 4 HLA A, B, C and DR-matched stimulator-responder pairs utilising both the OKT3 and the cytokine allostimulation techniques. Again, TCR Vβ sub-family distribution in $CD4^+$ and $CD8^+$ $CD69^-$ responder cells after 72 hours of allostimulation closely resembled that of baseline responder cells using both techniques of allostimulation, whereas skewing of TCR Vβ sub-family distribution was seen in $CD69^+$ responders following allostimulation. Skewing of $CD69^+$ responder cell TCR Vβ sub-family distribution was more marked in $CD8^+$ than $CD4^+$ alloresponders. Furthermore, different TCR Vβ sub-families were over-represented in $CD69^+$ alloresponder cells after allostimulation utilising the OKT3 and the cytokine allostimulation technique. The modular TCR Vβ sub-family frequency pertubation from baseline values are shown in Table 5. 7. TCR Vβ sub-family distribution in baseline cells and $CD69^-$ and $CD69^+$ responders after allostimulation (using both techniques) in a representative HLA-matched pair is shown in Figure 5 6.

Allostimula -tion technique	**Responder Cells**	**Modular TCR Vβ frequency perturbation, % from baseline value (Mean+/-standard deviation)**		**P value in paired two-tailed Student's t-test**
		$CD69^-$	**$CD69^+$**	
OKT3	**$CD4^+$**	*19.9+/-8.6*	*35.6+/-16*	*Ns*
	$CD8^+$	*24.6+/-9.2*	*79.9+/-41*	*0.04*
Cytokine	**$CD4^+$**	*21.5+/-5.5*	*64.4+/-22*	*0.03*
	$CD8^+$	*34.9+/-18*	*79.9+/-9.2*	*0.02*

Table 5.6 Perturbation from baseline frequencies of TCR Vβ sub-families in $CD69^-$ and $CD69^+$ responder cells after HLA-matched allostimulation.

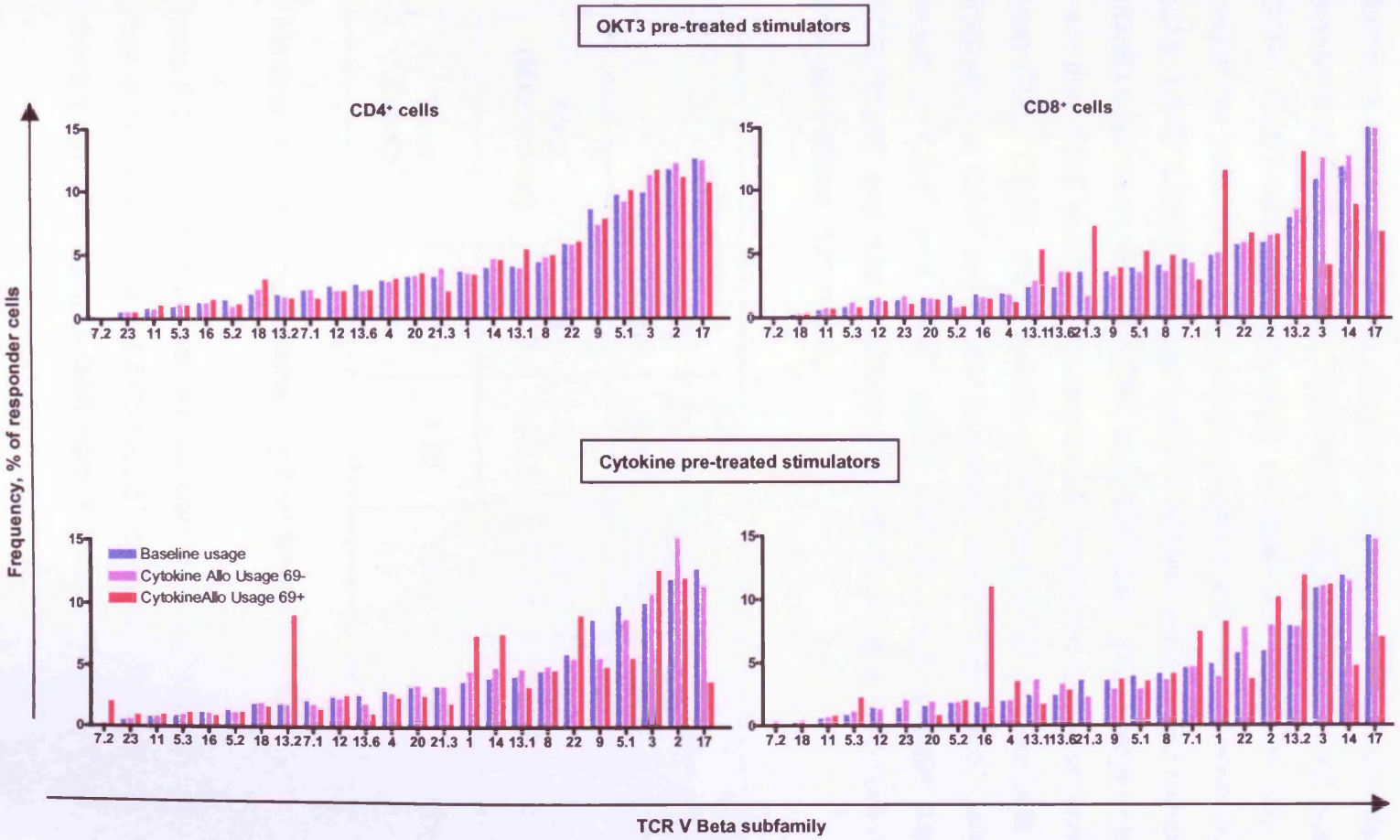

Figure 5.6 TCR Vβ sub-family distribution in baseline, $CD69^-$ and $CD69^+$ responder cells after 72 hours allostimulation in the MLR with HLA-matched OKT3 or cytokine pre-treated stimulator cells. Results are shown for $CD4^+$ and $CD8^+$ cells in a representative stimulator-responder pair. TCR Vβ sub-families are plotted in ascending frequency in baseline responder cells.

5.4.4 Co-expression of CD69 and CD25 on Alloreactive T cells

Three subsets of alloreactive cells in both $CD4^+$ cells and $CD8^+$ cells could be identified by their expression of the two activation antigens CD69 and CD25 following allostimulation by HLA-mismatched irradiated stimulator cells in the standard MLR. These are $CD69^+CD25^-$, $CD69^+CD25^+$ (dual positive) cells and $CD69^-CD25^+$ cells. Considerable variation was seen in the percentage of responder cells expressing each activation antigen between HLA-mismatched pairs. $CD69^+CD25^-$responder cells appeared earliest and exhibited the lowest maximal frequency in both $CD4^+$ and $CD8^+$ cells (with a larger response in $CD4^+$ cells than $CD8^+$ cells) Dual positive cells appeared later and were more frequent than $CD69^+CD25^-$ cells in both $CD4^+$ and $CD8^+$ cell subsets with a greater frequency in $CD4^+$ cells. Last to appear were $CD69^-CD25^+$ cells (in equivalent levels in $CD4^+$ and $CD8^+$ cells) and in much greater frequencies than $CD69^+CD25^-$ and dual positive cells, not reaching a maximum level by the last time point tested (120 hours).

	$CD4^+$			**$CD8^+$**		
	$CD69^+$ $CD25^-$	**$CD69^+$ $CD25^+$**	**$CD69^-$ $CD25^+$**	**$CD69^+$ $CD25^-$**	**$CD69^+$ $CD25^+$**	**$CD69^-$ $CD25^+$**
Max (Mean+/-sd)	*3.8 +/-2.7*	*6.2 +/-3.6*	*22.2 +/-14*	*1.8 +/-1.6*	*2.5 +/-1.8*	*24.1 +/-17*
Time (hours)	*72*	*120*	*120**	*72*	*96*	*120**

* Maximal response not reached by final time point (120 hours)

Table 5.7 Mean maximal expression (Max) of $CD69^+CD25^-$, $CD69^+CD25^+$ (dual positive) cells and $CD69^-CD25^+$ cells on HLA-mismatched responders (above autologous control) (and median time to maximal expression).

These three distinct subsets of both $CD4^+$ and $CD8^+$ responder cells ($CD69^+CD25^-$, $CD69^+CD25^+$ (dual positive) cells and $CD69^-CD25^+$ cells) appeared with the same temporal relationship following stimulation with

irradiated HLA A-, B-, C- and DR-matched (or single antigen mismatched) OKT3 pre-treated stimulator cells. Maximal $CD69^{-}CD25^{+}$ expression occurred earlier than after HLA-mismatched allostimulation. All three responder cell subsets were also identified following allostimulation with cytokine pre-treated HLA-matched stimulators, albeit at much lower frequencies than following allostimulation with OKT3 pre-treated stimulators, and responses above autologous controls were not seen in all matched pairs. In responders where CD69 and CD25 expression was seen (above that seen with autologous stimulators) the temporal relationship of the three cell subsets was similar to that seen after HLA-mismatched allostimulation, but maximal expression of $CD4^{+}CD69^{+}CD25^{-}$ cells occurred later (at 96 hours). (Table 5.8 and Figures 5.7 and 5.8).

Allostimulation technique		$CD4^{+}$			$CD8^{+}$		
		$CD69^{+}$ $CD25^{-}$	$CD69^{+}$ $CD25^{+}$	$CD69^{-}$ $CD25^{+}$	$CD69^{+}$ $CD25^{-}$	$CD69^{+}$ $CD25^{+}$	$CD69^{+}$ $CD25^{+}$
OKT3	Max	*11.7 +/-5.6*	*21.6 +/-7.0*	*39 +/-15*	*2.8 +/-1.3*	*5.5 +/-4.2*	*31 +/-21*
	Time (hours)	*72*	*72*	*96*	*72*	*72*	*96*
Cytokine	Max	*1.8 +/-1.7*	*1.8 +/-1.7*	*8.5 +/-6.6*	*0.9 +/-0.9*	*0.5 +/-1.0*	*5.7 +/-8.8*
	Time (hours)	*96*	*120*	*72*	*72*	*72*	*120*

Table 5.8 Mean maximal expression (Max) (+/- standard deviation) of $CD69^{+}CD25^{-}$, $CD69^{+}CD25^{+}$ (dual positive) and $CD69^{-}CD25^{+}$ cells on HLA-matched responders (above that seen on autologous control cells) following allostimulation with OKT3 or cytokine pre-treated stimulators (and median time to maximal expression).

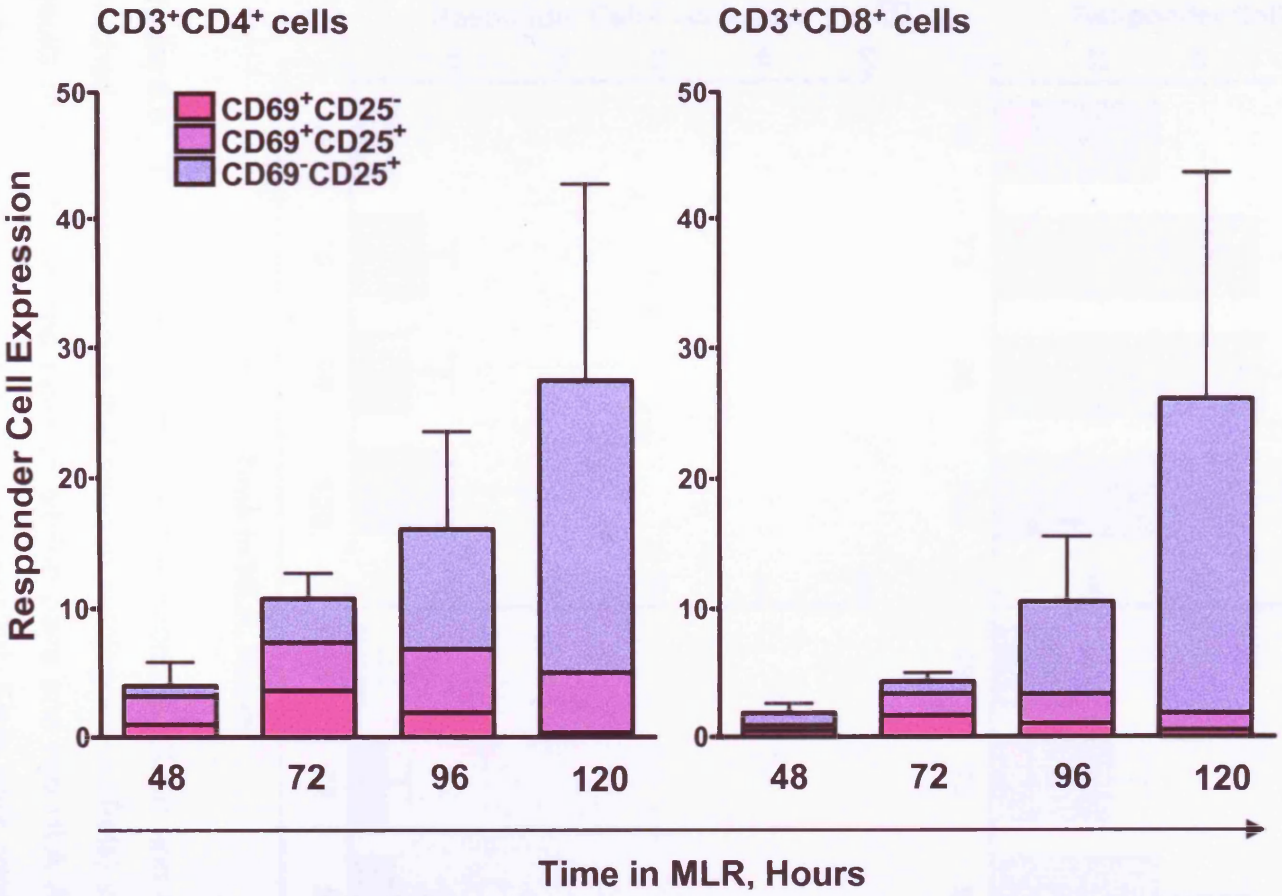

Figure 5.7 Expression of the activation markers CD69 and CD25 on HLA-mismatched responders (above that seen in autologous controls) in a standard MLR. The results of 7 mismatched pairs are presented. Error bars represent standard deviation of the total percentage of responder cells positive for CD69 and/or CD25.

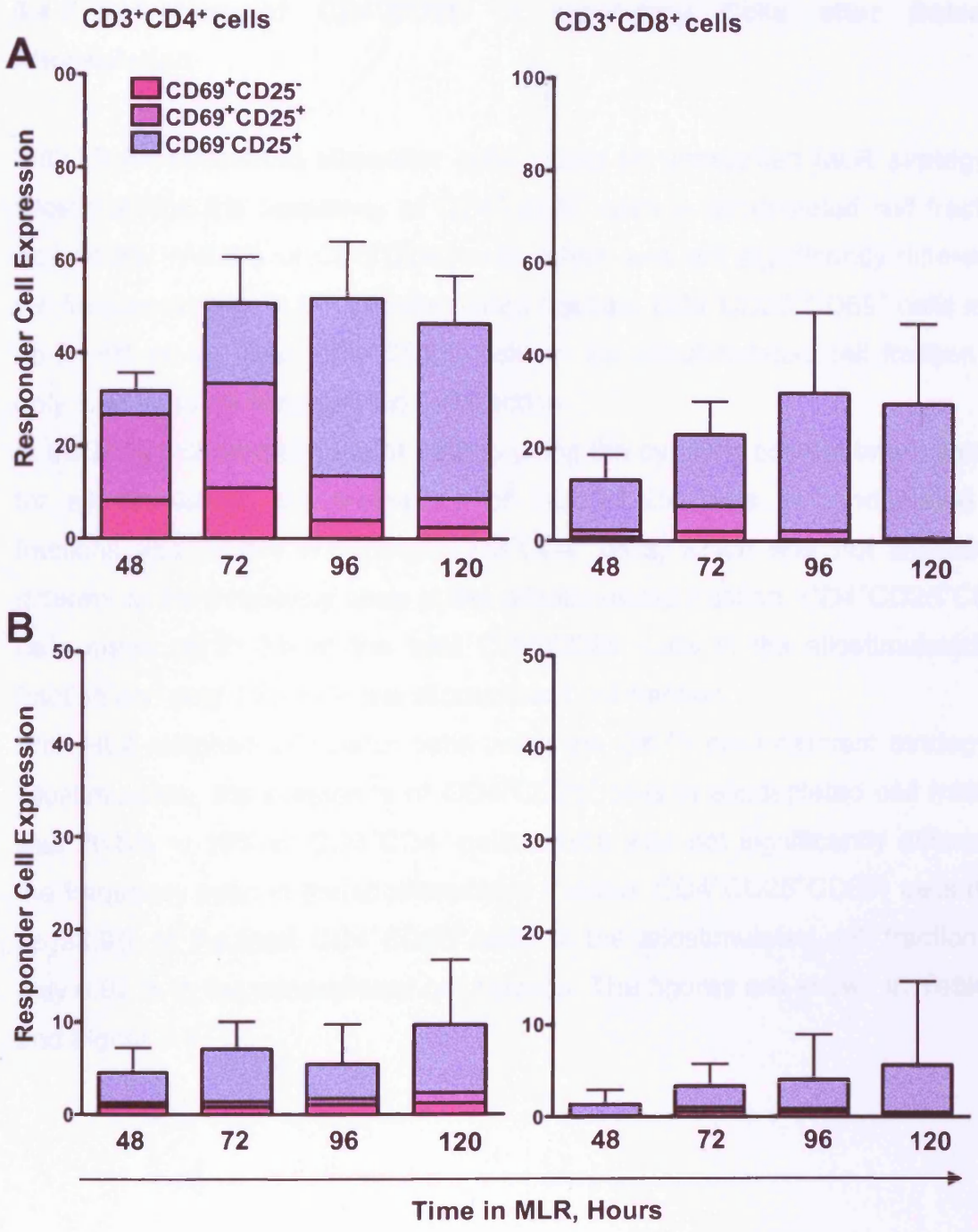

Figure 5.8 Expression of the activation markers CD69 and CD25 on HLA-matched responders (above that seen in autologous controls) in the MLR. The results of 7 HLA-matched pairs (5 sibling pairs and two HLA A, B, C and DR matched unrelated donor pairs) are presented. Error bars represent standard deviation of the total percentage of responder cells positive for CD69 and/or CD25. Results are shown for the same 7 pairs using;
(A) OKT3 pre-treated stimulators. (B) Cytokine pre-treated stimulators.

5.4.5 Retention of $CD4^+CD25^+$ T regulatory Cells after Selective Allodepletion

With HLA-mismatched stimulator cells, using an unmodified MLR strategy for allostimulation the frequency of $CD4^+CD25^+$ cells in allodepleted cell fractions was 14.9% +/-3.6% of $CD3^+CD4^+$ cells, which was not significantly different to the frequency seen in the allostimulated fraction. $CD4^+CD25^+CD69^+$ cells made up 24.6% of the total $CD4^+CD25^+$ cells in the allostimulated cell fraction and only 1.69 % in the allodepleted cell fraction.

In the HLA-matched stimulator setting using the cytokine pre-treatment strategy for allostimulation, the frequency of $CD4^+CD25^+$ cells in allodepleted cell fractions was 26.8% +/-13% of $CD3^+CD4^+$ cells, which was not significantly different to the frequency seen in the allostimulated fraction. $CD4^+CD25^+CD69^+$ cells made up 21.3% of the total $CD4^+CD25^+$ cells in the allostimulated cell fraction and only 1.83 % in the allodepleted cell fraction.

With HLA-matched stimulator cells using the OKT3 pre-treatment strategy for allostimulation, the frequency of $CD4^+CD25^+$ cells in allodepleted cell fractions was 76.8% +/-19% of $CD3^+CD4^+$ cells, which was not significantly different to the frequency seen in the allostimulated fraction. $CD4^+CD25^+CD69^+$ cells made up 33.9% of the total $CD4^+CD25^+$ cells in the allostimulated cell fraction and only 0.92 % in the allodepleted cell fraction. The figures are shown in Table 5.9 and Figure 5.9.

$CD3^+CD4^+$ cells	HLA-mismatched stimulators			HLA-matched OKT3 pre-treated stimulators			HLA-matched cytokine pre-treated stimulators		
	AC	Pre-depletion	Post-depletion	AC	Pre-depletion	Post-depletion	AC	Pre-depletion	Post-depletion
$CD25^+CD69^-$	8.8+/-3	12+/-5	15+/-4	15+/-6	39+/-18	76+/-18	14+/-2	20+/-3	26+/-13
$CD25^+CD69^+$	0.90+/-0.6	3.4+/-1.8	0.20+/-0.2	0.60+/-0.03	18+/-0.9	0.75+/-0.5	1.3+/-0.85	7.4+/-10	0.40+/-0.15
$CD25^-CD69^+$	0.80+/-0.3	3.6+/-0.7	0.20 +/-0.3	6.6 +/-8.4	16.3+/-7.2	0.6 +/-0.4	1.2+/-0.9	2.3 +/-2.6	0.2 +/-0.2
Total $CD25^+$	9.7+/-2.9	15+/-4.4	15+/-3.6	15+/-9	57+/-16	77+/-19	15+/-2.8	27+/-13	27+/-13
$CD25^+CD69^+$ % of total $CD25^+$	11+/-8	25+/-15	1.7+/-2	4.8+/-3	34+/-11	0.92+/-0.5	8.2+/-4	21+/-25	1.8+/-1.5

Table 5. 9 $CD4^+CD25^+$ $CD69^-$, $CD4^+CD25^+$ $CD69^+$ and total $CD4^+CD25^+$ responder cells after allostimulation and selective allodepletion Results of 12 fully HLA-mismatched pairs (of which 7 were selectively allodepleted) and two HLA-matched pairs are presented as mean +/- sd. AC=Autologous Controls

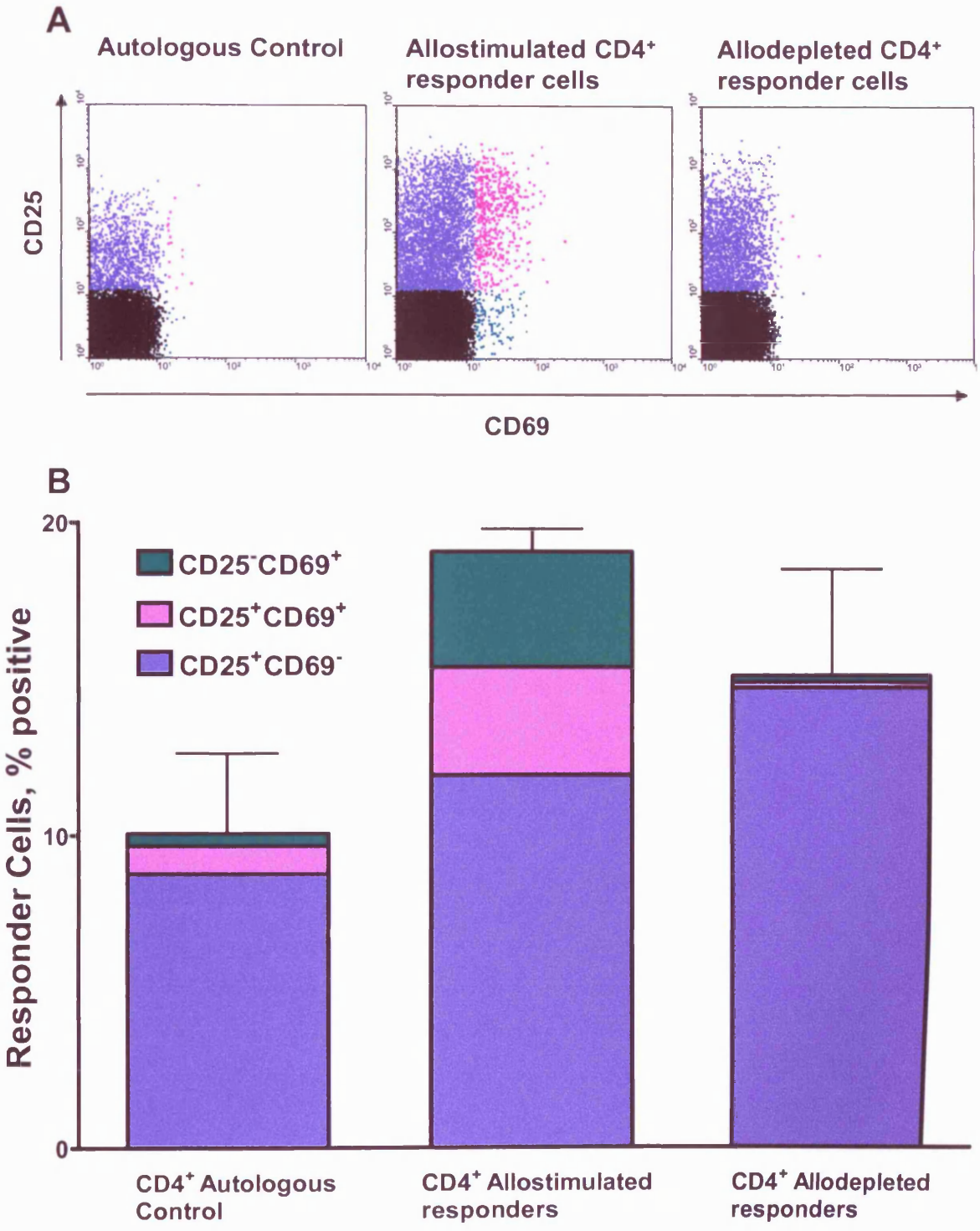

Figure 5. 9 $CD3^+CD4^+CD25^-CD69^+$, $CD3^+CD4^+CD25^+CD69^+$ and $CD3^+CD4^+CD25^+CD69^-$ cells before and after selective allodepletion of $CD69^+$ cells;

(A). A representative dot plot of HLA-mismatched $CD3^+CD4^+$ responders after 72 hours in a standard MLR.

(B). Mean frequency in 12 HLA-mismatched pairs. Error bars represent standard deviation of total $CD3^+CD4^+CD25^+$ cell frequency.

Two HLA-mismatched donor-recipient pairs were used to confirm the retention of *functional* $CD4^+CD25^+$ T regulatory cells after selective allodepletion following the experimental schema outlined in Figure 5.3.

Proliferative responses after 120 hours in a standard MLR with third party HLA-mismatched stimulator cells were reduced to 20%+/-12% of the value seen with unmanipulated responder cells when an equal number of sorted $CD4^+CD25^+$ cells (>80% pure) from selectively allodepleted responders were added to unmanipulated responder cells ($p=0.005$ in a paire two-tailed Student's t test). The addition of non-$CD4^+CD25^+$ cells from selectively allodepleted responders did not significantly alter proliferative responses in this setting (Figure 5. 10).

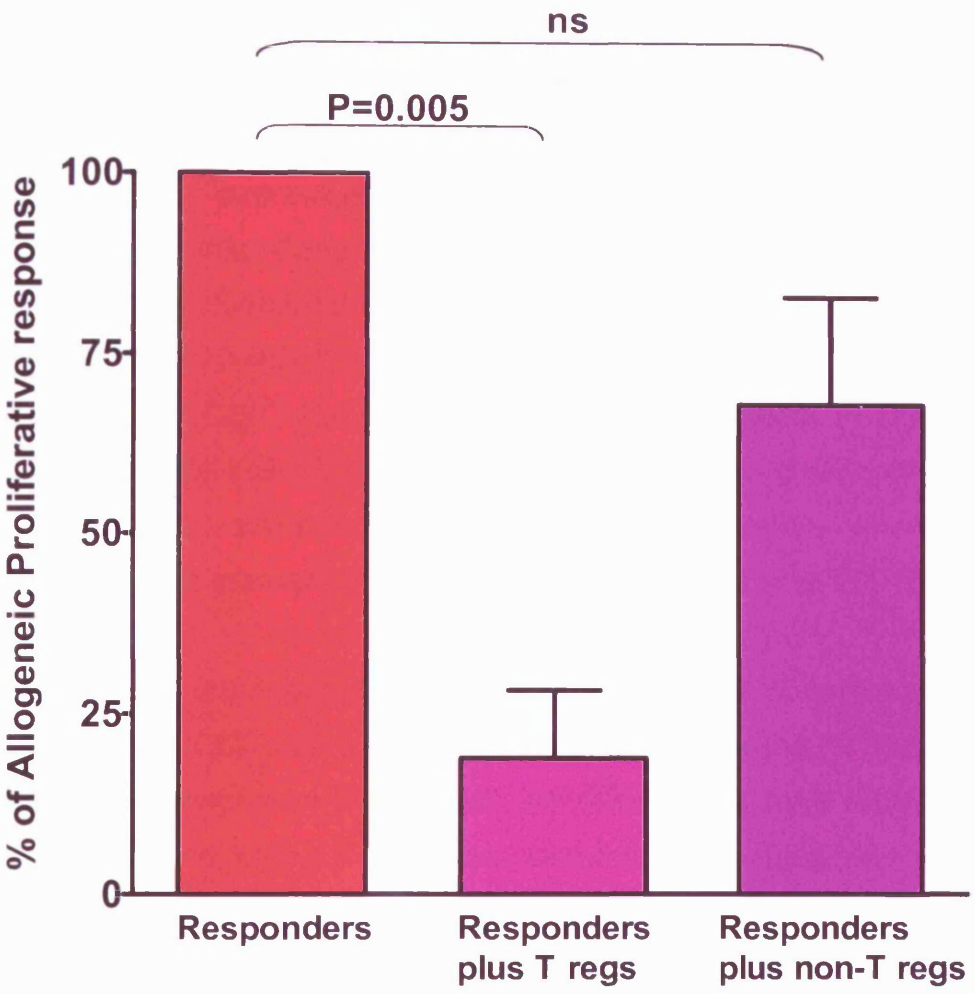

Figure 5.10 $CD4^+$ $CD25^+$ T regulatory cells present in selectively allodepleted cells retain the ability to suppress proliferative responses to third party HLA-mismatched stimulator cells. Results are the mean (+/- standard deviation) of triplicate proliferation assays in two HLA-mismatched pairs.

5.5 Chapter Discussion

The normal ranges for naïve and memory phenotypes of $CD4^+$ and $CD8^+$ T cells (based on their expression of CD45RA and CCR7) have been established in healthy individuals. Considerable heterogeneity was seen between individuals but the ranges described are in keeping with those published in the literature. [Sallusto *et al.* 2004b] The relative proportions of T_{CM} and T_{EM} in blood vary in the $CD4^+$ and $CD8^+$ compartments; T_{CM} is predominant in $CD4^+$ and T_{EM} in $CD8^+$. Within the tissues, however, T_{CM} and T_{EM} show characteristic patterns of distributions. T_{CM} are enriched in lymph nodes and tonsils, whereas lung, liver, and gut contain greater proportions of T_{EM}.[Campbell *et al* 2001;Morimoto *et al.* 1985]

The naïve and memory phenotypes of $CD4^+$ and $CD8^+$ T cells were examined in baseline and $CD69^+$ alloreactive responder cells following 72 hours of HLA-mismatched stimulation in the MLR. All cell subsets were represented in both $CD4^+CD69^+$ and $CD8^+CD69^+$ responder cells in keeping with published data that both naïve and memory T cells are able to proliferate in response to alloantigenic stimulation in the MLR.[Morimoto *et al* 1985].

The presence of alloreactivity in both the $CD45RA^+$ and $CD45RO^+$ T cell populations is likely to be a manifestation of both intrathymic and post-thymic T cell selection and development. Alloreactive T cells can be detected in the circulation of individuals with no prior exposure to alloantigens (except possibly *in utero*).[Ryser and MacDonald 1979;van Oers *et al.* 1978] and initial publications indicated frequencies of responding T cells of between 1 and 5% to any given allogeneic haplotype,[Lindahl and Wilson 1977a;Lindahl and Wilson 1977b] although recent reports investigating precursor frequency of alloreactive helper and cytotoxic T lymphocytes by limiting dilution assays have shown much lower frequencies.[Wang *et al.* 1996;Young *et al.* 1998]

The data presented demonstrates over-representation of T_{EM} cells in the $CD69^+$ alloresponding cell fraction, (with a significant increase compared to baseline frequency in the % of $CD4^+$ $CD69^+$ T_{EM} cells after 72 hours of HLA-mismatched allostimulation (and a trend towards increased frequency of T_{EM} in $CD8^+CD69^+$ cells). Although prolonged antigenic stimulation leads to an antigen-driven shift in the isoform of CD45 expressed on T cells (from RA to RO) this process takes several rounds of cell division (e.g 6-7 days of dendritic cell alloantigen

stimulation for $CD8^+$ cells).[Geginat *et al.* 2003] Thus the relative increase in T_{EM} cells in $CD69^+$ alloresponders is likely to represent a true over-representation of this cell subset within the pool of T cells able to respond to allostimulation, (rather than a shift of phenotype of alloresponders from naïve to memory cells). In order to prove this, experiments measuring the CD69 expression, proliferation and cytokine secretion in response to allostimulation of sorted T cell memory and effector subsets could be performed.

There is conflicting data in the literature regarding the differential capacity for memory and effector T cell subsets to mount an alloreactive response and the responses may be different for $CD4^+$ and $CD8^+$ T cells.

Murine data have shown that alloreactive responses were mediated mainly by naïve ($CD62L^+$) T cells, and that the transfer of allogeneic memory ($CD62L^-$) T cells contributed to immune reconstitution without GvHD in irradiated recipient mice.[Chen *et al.* 2004a] Foster et al have recently demonstrated that human memory ($CD62L^-$) T cells were less responsive to allogeneic stimuli than $CD62L^+$ naïve T cells.[Foster *et al.* 2004a] However, In vitro alloresponses do not appear to be limited exclusively to $CD45RA^+$ naïve T cells. O' Brien et al reported responder proliferation after stimulation in the MLR with both purified $CD45RA^+$ and $CD45RO^+$ responders. Greater proliferation was observed with $CD4^+CD45RO^+$ cells than with $CD4^+CD45RA^+$ cells, whereas $CD8^+CD45RA^+$ responders proliferated significantly more in response to allostimulation than $CD8^+CD45RO^+$ responders.[O'Brien and Kemeny 1998]

Recipients of AHSCT who develop chronic GvHD have an increased frequency of circulating $CD4^+$ T_{EM} cells.[Yamashita *et al* 2004] The pivotal role of memory T cells in the pathogenesis of GvHD has also recently been confirmed in a murine GvHD model where blockade of the CCR7 receptor led to reduced chronic GvHD. Further work needs to examine the naïve/memory phenotype of CD69 alloreactive and non-alloreactive T cells in HLA-matched allostimulation using different strategies of potentiating allostimulation, and with the assessment of expression of other molecules used to differentiate T cell subsets (CD62L, CD27,CD28).

TCR Vβ sub-family distribution in $CD69^+$ responder cells following HLA-mismatched allostimulation demonstrated significantly more perturbation from baseline frequencies in $CD8^+$ responders. Increases in frequencies of 2-4 TCR Vβ sub-families were seen with corresponding decreases in other sub-families.

This may represent an oligoclonal response to alloantigens, (although expansions of discrete TCR Vβ sub-families does not necessarily imply responses to different alloantigens). A single MHC-peptide combination can positively select thymocytes bearing many distinct TCRs (albeit based on interactions with relatively low avidity). [Chmielowski *et al.* 1999;Ignatowicz *et al.* 1996] Restricted (and conserved) T cell repertoires have been identified amongst PBMC pools from healthy blood donors responding to irradiated PBMCs disparate for a single HLA class II molecule in an MLR. [De Palma and Gorski 1995] Importantly, these data demonstrated that the TCR Vβ sub-family restriction was conserved across different individuals to a given HLA class II antigen, whereas different responses were seen to different HLA Class II alloantigens. Subsequently much evidence has emerged for restricted TCR Vβ distribution in cells mediating GvHD. Infiltrating T cells during liver graft-versus-host disease show a restricted T cell repertoire,[Margolis *et al.* 2000] as do T cells present in skin and mucosal GvHD lesions in patients post AHSCT.[Kubo *et al.* 1996] Separation of anti-host and anti-tumour responses based on infusion of $CD4^+$ T cells with selected TCR Vβ sub-family restriction known to be associated with either a strong alloresponse or a strong anti-tumour response has been demonstrated in a murine model.[Patterson and Korngold 2001]

TCR Vβ sub-family distribution in the $CD69^-$ non-alloresponding cell fraction closely resembled that of baseline responder cells, indicating that a broad spectrum of TCR Vβ sub-families would be maintained in the non-alloreactive $CD69^-$ cell fraction following selective allodepletion and that the clinical use of such cell fractions might maintain potential for broad spectrum immune reconstitution.

Following HLA-matched allostimulation qualitatively similar results were observed. It is of note that the use of different techniques for potentiating allostimulation in the HLA-matched setting (cytokine or OKT3 pre-treatment of stimulators) *within* individual matched pairs led to different patterns of TCR Vβ sub-family distribution in $CD69^+$ responder cells, with increases in some sub-families common to both allostimulation techniques, but increases in other TCR Vβ sub-families limited to one technique. This observation may represent differential expression of mHags on myeloid or lymphoid APCells.

Alloreactive responses to mHags in the MHC-matched setting have only been recently defined. Much work has been published in murine H2-matched models. The immunodominant $CD4^+$ TCR Vβ sub-family repertoires involved in GvHD responses to mHags have been described in such murine models, and heterogeneity has been described of $CD4^+$ T cell responses to mHags, and correlation with epithelial tissue infiltrates. Repertoire analysis of $CD8^+$ T cell responses to mHags involved in GvHD has also been reported, with restriction in GvHD-mediating cells suggesting oligoclonal T cell responses to a limited number of murine mHags. [Berger and Korngold 1997;Friedman *et al.* 1998;Friedman *et al.* 2001;Jones *et al.* 2004] Maccario et al have recently analysed TCR repertoire by CDR3 size spectratyping before and after selective allodepletion of HLA-mismatched responder cells based on CD69 expression and subsequent in vitro anti-CTLA-4 antibody.[Maccario *et al.* 2003] Only presented in abstract form, this work reported polyclonal TCR Vβ repertoire in MLR cultures. However it is not clear how live responder DNA was differentiated from DNA from irradiated stimulator cells (or whether alloreactive cells were further identified by any means), a criticism common to the work published by De Palma et al demonstrating restricted responder cell TCR Vβ sub-family usage in responder cells in the MLR.[De Palma and Gorski 1995]). Thus the degree of TCR Vβ repertoire restriction in human alloreactive cells, particularly in the setting of either multiple HLA-mismatches or in the HLA-matched setting remains to be accurately described.

Characterisation of TCR Vβ sub-family distribution in responder alloreactive cells identified by CD69 expression might aid the development of strategies to raise non-alloreactive cells with specific anti-leukaemic activity or antiviral activity. This potential application is discussed in more detail in Chapter 6.

Three distinct populations of responder cells appear following both HLA-mismatched and HLA-matched allostimulation based on expression of CD69 and CD25. The $CD69^+$ $CD25^-$ response occurs first (maximum at 24-48 hours), followed by dual positive cell response (maximum at 48-72 hours) and lastly the $CD69^-$ $CD25^+$ cell response (maximum at 96-120 hours). Only a proportion of alloreactive cells expressed both activation markers at any given time point. It remains to be determined whether the $CD69^-$ $CD25^+$ cells upregulate CD25 as a response to cytokines secreted by $CD69^+$ cells or as a response to direct

alloantigen stimulation. Trans-well plate or PKH-26 cell labelling experiments would be needed to resolve this issue. It is also unknown whether $CD69^+$ and $CD25^+$ alloreponsive cells are predominantly two different cellular subsets or represent the expression of different antigens sequentially on the same alloreactive cells. To determine this alloresponders could be sorted by flow cytometry based on their expression of CD69, labelled with PKH-26 and returned to the MLR, and the expression of CD25 on $PKH\text{-}26^+$ cells subsequently measured. Selective allodepletion of responder cells based on the expression of both activation markers at the same time point might improve the efficiency of allodepletion above that seen with depletion based on expression of either activation antigen alone. This approach was undertaken by Fehse et al who found selective immunomagnetic depletion of $CD69^+$ and $CD25^+$ HLA-mismatched responder cells was more effective that selective depletion based on either antigen alone.[Fehse *et al* 2000a] It has recently been shown that $CD4^+CD25^+$ T regulatory cells are hyporesponsive to allostimulation and therefore these cells are unlikely to upregulate CD69 expression upon allostimulation and become $CD4^+$ $CD25^+$ $CD69^+$ cells.[Browning M.B *et al.* 2003] Frequencies of $CD4^+CD25^+$ cells were retained in CD69-selectively allodepleted cell fractions. $CD4^+CD25^+$ cells from allodepleted cell fractions retained their immunosuppressive function in allogeneic MLRs when added in a 1:1 ratio to autologous responder cells. The role of $CD4^+CD25^+$ T regulatory cells in suppressing alloreactivity is increasingly being recognised and the loss of such cells from the donor pool might result in increased GvHD. Loss of T regulatory function is seen in individuals with autoimmune and atopic conditions.[Browning *et al* 2003;Papiernik 2001] Non-selective T cell depletion leads to disordered immune reconstitution and the loss of hierarchical control, which may be manifest as autoimmune disorders such as autoimmune haemolytic anaemia.[Drobyski *et al.* 1996]

MHC disparate murine models also support the ability of $CD4^+CD25^+$ T regulatory cells to suppress GvHD whilst retaining GvL responses against leukaemia and lymphoma.[Edinger *et al.* 2003;Hoffmann *et al.* 2002;Johnson *et al.* 2002;Jones *et al.* 2003] There is however a discrepancy between the murine data and emerging evidence from the human AHSCT setting. In a study by Clark et al, patients with chronic GVHD after AHSCT had markedly elevated numbers of $CD4^+CD25^{high}$ T cells as compared to patients without GVHD.

Although $CD4^+CD25^{high}$ T cells derived from patients in both groups were of donor origin, these cells may be present in higher frequencies as a result of (or response to) the GvHD.[Clark *et al.* 2004] Importantly, Stanzani et al have reported an increase in $CD4^+CD25^+$ T regulatory cells in the graft inoculum of patients who subsequently developed GvHD.[Stanzani *et al.* 2004] Thus it seems the evidence of the role of $CD4^+CD25^+$ T regulatory cells in suppressing human alloresponses whist retaining GvL responses has yet to be demonstrated. Nevertheless attempts have been made to purify human donor $CD4^+CD25^+$ T regulatory cells that show antigen-specificity for recipient alloantigens with the therapeutic aim of suppressing alloreactive responses in vivo.[Trenado *et al.* 2004]

Selective allodepletion based upon the upregulation of CD25 will remove (or destroy) not only alloreactive cells that have upregulated CD25 upon allostimulation but any cells that express CD25 constitutively including $CD4^+CD25^+$ T regulatory cells: such techniques will therefore deplete the donor T cell pool of the T regulatory compartment with the potential of increased alloreactivity and disordered immune reconstitution.

Chapter 6 Expression of CD69 on CMV-reactive Cells

6.1 Introduction

Evidence is emerging that any given TCR may interact with more than one MHC/peptide combination and in fact may be able to bind a variety of MHC/peptide combinations with different levels of affinity. The interaction between TCR and MHC-peptide shows degeneracy for both MHC class I-CD8 and MHC class II-CD4 interactions and a single TCR is able to recognize structurally distinct MHC-peptide complexes.[Goldrath and Bevan 1999;Mason 1998;Tallquist *et al.* 1996]

Although previously thought that rare microbial antigens with peptide sequences closely related to self-peptides could represent mimics (and thus be responsible for the induction of autoimmune diseases), it has now been shown that TCRs can share affinity for structurally diverse antigenic molecules.[Wucherpfennig 2004] There are many animal data supporting a link between CMV infection and GvHD. Murine models have suggested that early virus-induced immune responses may promote the development of severe graft-versus-host responses, including the enhancement of donor antihost specific cytotoxic T cells.[Cray and Levy 1990] [Jones *et al.* 1996] The mechanism of exacerbation of murine GvHD caused by the induction of CMV disease remains to be elucidated and may not be limited to shared T cell affinity for CMV and alloantigens. The effect of CMV infection on worsening acute GvHD in mice was abrogated by depletion of host $CD4^+$ cells (but not $CD8^+$ cells) supporting the hypothesis that CMV-activated $CD4^+$ T cells enhance the production of a donor anti-host MHC Class I $CD8^+$ response.[Cray and Levy 1993]

An association between CMV infection and GvHD in humans was first suggested by a case series published in 1984 describing 68 consecutive allogeneic bone marrow transplant patients.[Lonnqvist *et al.* 1984] Chronic GVHD was significantly more common among patients who had previously experienced CMV infection with a median time of onset of 128 days prior to GvHD Other series later confirmed this finding.[Jacobsen *et al.* 1986;Ringden *et al.* 1985] Much evidence has been published demonstrating an association between CMV infection and acute GvHD, although in this setting clinically apparent CMV infection typically occurs 2-4 weeks after the onset of acute

GvHD (and may be more closely related to immunosuppressive therapy used to control it).[Miller *et al.* 1986]

Recipients of non-T depleted marrow transplants for (both myeloid and lymphoid) acute leukaemia who developed CMV infection were at a significantly lower risk of leukaemic relapse (independent of the occurrence of either acute or chronic GvHD) indicating that donor T cells may share affinity for CMV antigens and leukaemia restricted or leukaemia-specific antigens.[Jacobsen *et al.* 1987;Jacobsen *et al.* 1990;Lonnqvist *et al.* 1986] In a series of 103 consecutive patients who had leukaemia and who received non T-depleted bone marrow transplants from HLA-matched sibling donors, donor CMV seropositivity was shown to significantly improve overall survival due to lower relapse incidence in HLA-A*0201$^+$ (but not HLA-A*0201$^-$) recipients. Donor CMV seropositivity was associated with chronic GvHD but, even in patients without chronic GvHD, donor CMV seropositivity significantly improved survival.[Nachbaur *et al.* 2001], supporting a hypothesis of HLA A*0201-restricted donor T cells with shared affinity for CMV antigens and mHags over-expressed on leukaemia cells.

Recently large meta-analyses have been published from data derived from international registries and somewhat conflicting results have been reported. One series reporting over 6000 unrelated donor transplants found no effect of donor CMV seropositivity on acute or chronic GvHD, relapse or overall mortality,[Kollman *et al.* 2001] whereas another similar sized series found that donor CMV seropositivity decreased overall mortality (but did not independently affect rates of GvHD or relapse) in unrelated (but not sibling) transplants.[Ljungman *et al.* 2003b] It must be stressed that these series report the effects only of CMV donor serostatus (and not of subsequent CMV disease), and derive data from recent periods where molecular monitoring and pre-emptive treatment have reduced the incidence of CMV disease considerably. Indeed reduced risk for extensive chronic graft-versus-host disease in patients receiving transplants with human leukocyte antigen-identical sibling donors given pre-emptive anti-CMV therapy (based on CMV DNA PCR monitoring) been reported.[Larsson *et al.* 2004]

The development of HLA Class I restricted CMV-peptide tetramers has allowed the measurement of CD8$^+$ cellular CMV-specific immune responses to CMV after allogeneic transplantation.[Aubert *et al* 2001] However staining with CMV-

peptide tetramers in this setting does not identify whether the $CD8^+$ T cells originate from the donor or from recipient cells, and does not distinguish the individual T cell clones that comprise the tetramer positive population. The reconstitution of CMV-specific $CD8^+$ T cells at the level of individual clones following AHSCT using clonotypic probing has been described.[Gandhi *et al.* 2003] Following CMV IgG $Donor^+/Recipient^-$ AHSCT, donor CMV-specific clones were not detectable in two of three CMV seronegative recipients whereas in a third (CMV IgG $Donor^+$) CMV IgG^- HLA-$A*0201^+$ recipient and who experienced severe acute GvHD, a donor CMV-specific $CD8^+$ T cell clone was repeatedly detectable after transplantation. A possible explanation for the persistence of this donor CMV-specific $CD8^+$ T cell clone in the recipient is that its TCR may have been cross-reactive with another antigen in the recipient. In HLA-$A*0201^+$ recipients, donor-derived CMV-specific T cells may cross-react with HLA-A*0201-restricted recipient minor histocompatability antigens,[Nachbaur, Bonatti, Oberaigner, Eibl, Kropshofer, Gastl, Nussbaumer, Einsele, and Larcher 2001] raising the possibility donor CMV clones might be alloreactive and directly participate in the GvHD. Cwynarski et al.reported that CMV-peptide tetramer-positive $CD8^+$ T cells were detectable in only one of seven recipients following CMV IgG $Donor^+/Recipient^-$ AHSCT, and that this recipient was the only one of the seven to experience acute GvHD.[Cwynarski *et al.* 2001]

Local tissue infection with CMV virus has been reported in acute intestinal GvHD (and in this setting antiviral treatment may lead to resolution of the histopathological changes of GvHD),[Einsele *et al.* 1994] but is not a universal finding.[Appleton *et al.* 1995]

T cell clones were generated from skin-infiltrating T lymphocytes in recipients of unrelated donor marrow (matched for HLA-A, -B, -DR, and -DQ but mismatched for –DP) with acute GVHD. Analysis of the clones showed that the majority of them lacked HLA specificity, including mismatched HLA-DP. However, mAb to HLA antigens blocked proliferation of the majority of the clones, indicating that the clones recognized HLA-associated molecules. A few of the clones revealed augmented proliferation in the presence of CMV antigens and a few revealed cytolytic activity, supporting the hypothesis that human CMV antigens stimulate skin-infiltrating T lymphocytes present in acute GvHD.[Nikaein *et al.* 1994]

In donor-recipient pairs where donor T cells share affinity for CMV antigens and alloantigens, the selective removal of alloreactive cells might be expected to lead to a reduction in the frequency of cellular responses to CMV.

Following in vitro HLA-mismatched selective allodepletion of 4 HLA A*0201$^+$ individuals (mediated by anti-CD25 immunotoxin,) no reduction in frequency of HLA A*0201-NLV-Tetramer$^+$ cells was demonstrated, although 3 of 5 similar pairs tested showed a reduction in frequency (of 10-30%) of functional CMV-specific IFN-γ ELISpot-reactive CTLs.[Amrolia *et al* 2003a]

In mismatched MLRs the majority of CMV pp65 IFN-γ ELISPOT reactive cells are found within the responder CD69 ^{dull} (not the CD69$^-$) fraction of responder cells, suggesting that some CMV-specific responder cells become weakly activated by alloantigens in the MLR and could be removed by CD69-mediated depletion.[Nonn *et al.* 2003b] In a short series published in abstract form only, CMV IFN-γ ELISpot reactive cells were retained in some but not all cases following HLA-matched selective allodepletion utilising the cytokine modified MLR and immunomagnetic depletion of cells expressing CD25, CD69 and HLA DR.[Schumm *et al* 2003]

Results for selective allodepletion of HLA-mismatched and HLA-matched responders based on the expression of CD69 alone are presented in Chapter 4 of this thesis. Use of this strategy led to the retention of the majority, but not all of CMV CTLs in both the HLA-mismatched and HLA-matched setting. In fully HLA-mismatched pairs 88% of HLA A*0201-NLV peptide tetramer$^+$ cells were retained and 114% of functional cells by CMV-peptide-stimulated IFN-γ ELISpot. In matched pairs utilising the cytokine pre-treatment strategy of allostimulation 98% of HLA A*0201-NLV peptide tetramer$^+$ cells were retained and 89% of functional CMV-specific cells by peptide-stimulated IFN-γ ELISpot were retained.

However in HLA-matched pairs utilising OKT3 pre-treated stimulators only 77% of HLA A*0201-NLV peptide tetramer$^+$ cells and 72% of functional cells by CMV-peptide-stimulated IFN-γ ELISpot were retained following allodepletion. As the removal of only alloreactive cells (in the absence of cross-reactivity) would lead to relative concentration of CMV-specific CTLs in the allodepleted fraction, this loss of cellular CMV activity is perhaps under-estimated. The loss of functional CMV-specific cells is more marked than the loss of CMV tetramer$^+$

cells following HLA-matched allostimulation and selective allodepletion, and is in keeping with the data described by Amrolia et al following CD25 mediated allodepletion.[Amrolia *et al* 2003a] The greater degree of loss of functional CMV-specific cells in the HLA-matched setting suggests that there is greater cross-reactivity of alloreactive CTLs directed against mHags and CMV-specific antigens. Better preservation (albeit with greater variability) of third party activity post selective allodepletion was seen in HLA-matched pairs utilising OKT3 or cytokine pre-treated allostimulation (mean 94% and 101% respectively) than in HLA-mismatched allodepletion (mean 69%) which does not support the hypothesis that the loss of functional CMV-CTLs after HLA-matched selective allodepletion is a result of lower specificity of the allodepletion process.

The potential consequences of significant cross-reactivity between allogeneic antigens and CMV antigens would not be limited to the partial loss of CMV reactive CTLs after selective allodepletion. Considerable efforts have been made in recent years to raise CMV-specific donor CTLs for infusion post-AHSCT to aid recipient CMV recovery.[Sili *et al.* 2003;Walter *et al.* 1995] Two studies have shown that repeated rounds of *ex vivo* stimulation with CMV antigen (or peptide) may reduce alloreactivity as demonstrated by in vitro proliferation assays.[Einsele *et al.* 2002;Rauser *et al.* 2003] However the post-transplant infusion of small doses of donor derived CMV-specific CTLs (which underwent considerable *in vivo* expansion) caused Grade I skin GvHD in 3 of 16 recipients.[Peggs *et al.* 2003c] The use of larger cell doses or repeated administration may increase the risk of severe GvHD. Indeed in work recently presented in abstract form up to 10% of donor-derived CMV-specific $CD4^+$ cell clones intended for post-transplant infusion to aid recipient CMV immunity demonstrated significant proliferation in response to alloantigens.[Velardi 2003]

Various methods have been recently used to isolate CMV-specific T cells in immune individuals, including secretion of IFN-γ following simultaneous stimulation with CMV-specific MHC Class I-restricted peptides and CMV antigen,[Rauser *et al* 2003] and purification of CMV-specific $CD8^+$ T cells from peripheral blood using HLA-peptide tetramers.[Keenan *et al.* 2001] CMV antigen has been shown to stimulate CD69 expression on responding T cells in CMV IgG^+ healthy individuals.[Dunn *et al.* 2002] CD69 upregulation has also been used to identify Coxsackie virus B4-specific CTLs after incubation with infected cell lysate and TCR Vβ sub-family distribution of $CD69^+$ responder cells

were assessed by flow cytometry with antibodies to multiple TCR Vβ sub-family epitopes.[Varela-Calvino *et al.* 2001] Results presented in Chapter 4 of this thesis demonstrated restricted TCR Vβ sub-family distribution of alloreactive responder cells based on their expression of CD69. Expression of CD69 on CMV-specific CTLs after CMV-peptide stimulation might allow their identification and flow cytometric assessment of their TCR Vβ sub-family distribution, and any homology *within* individuals between CMV-specific and allospecific T cell TCR Vβ sub-family distribution might support the hypothesis of the existence of T cells within individuals with TCRs with shared affinity for CMV antigens and alloantigens.

6.2 Aims of the Experiments Described in this Chapter

1. To assess if CMV-specific CTLs could be identified by CD69 expression after NLV peptide stimulation in HLA A*0201$^+$CMV IgG$^+$ individuals;
2. To determine whether the frequencies of NLV peptide-stimulated CD69$^+$ CTLs correlated with the frequency of HLA A*0201-NLV-Tetramer$^+$ cells and/or to alternative functional assays of CMV CTL frequency (including IFN-γ ELISpot and intracellular IFN-γ generation);
2. To investigate whether NLV peptide-stimulated CD69$^+$ CTLs demonstrated any evidence of selective TCR Vβ sub-family usage within and/or between HLA A*0201$^+$CMV IgG$^+$ individuals;
3. To examine any potential homology of TCR Vβ sub-family usage between NLV peptide-stimulated CTLs and HLA-matched allostimulated CTLs within HLA A*0201$^+$ individuals that might support the hypothesis that cross-reactivity of antigen-specificity exists in such CTLs.

6.3 Materials and Methods

16 healthy volunteers who had past exposure to CMV were identified by the presence of CMV IgG by serological testing (either at the Department of Virology, Royal Free Hospital, London, or at registered transplant donor centres). Allelic typing of HLA Class I and Class II was performed on all individuals at the Anthony Nolan Research Institute.

Up to 10^6 PBMCs were suspended at a concentration of 10^6/ml in complete medium (with AB serum) in sterile plastic 12 x 75mm polystyrene tubes (Nunc) or polypropylene tubes (Kendall-Elkay) and stimulated with the HLA A*0201-restricted CMV pp65 derived nonamer NLVPMVATV (NLV) (Proimmune) or a CMV dummy peptide (restricted to an HLA Class I molecule not possessed by the individual). The optimal concentration of cells, amount of peptide and duration of stimulation was ascertained. CD69 expression on $CD3^+$, $CD3^+CD4^+$ and $CD3^+CD8^+$ cells was assessed by flow cytometry. A variety of CD69 antibodies was tested and assessed for sensitivity and specificity of $CD3^+CD8^+$ responses to NLV peptide.

All antibodies used were from BD and the staining protocol, event acquisition and analysis was as described in Chapter 2.2.1-2. The antibody panel used for flow cytometric assessment of basic cell phenotype before and after CMV-peptide stimulation/allostimulation is shown below:

Fluorochrome	**FITC**	**PE**	**PerCP**	**APC**
Antibody	CD3	CD4	CD8	CD69

Table 6.1 Antibody panel used for assessing CD69 expression on cell subsets after stimulation with NLV peptide.

The frequency of HLA A*0201-NLV peptide-specific CTLs was assessed by tetramer analysis on baseline cells (Chapter 2.2.3) and the frequency of functional HLA A*0201-NLV peptide-specific CTLs was assessed by IFN-γ ELISpot assay (Chapter 2.6). Intracellular generation of IFN-γ was measured using the Miltenyi APC-conjugated flow cytometric IFN-γ cytokine secretion assay detection kit (Chapter 2.7).

TCR Vβ sub-family distribution was assessed by flow cytometry using the IOTest BetaMark TCR Vβ Repertoire kit (Beckman Coulter, Chapter 5.3).

PBMCs from 6 HLA A*0201$^+$CMV IgG$^+$ individuals were pulsed with NLV peptide for 12 hours and the frequencies of TCR Vβ sub-families determined in CD8$^+$CD69$^+$ responders and CD8$^+$CD69$^-$ non-responders were measured. TCR Vβ sub-family frequencies were also assessed on fresh baseline cells.

Cytokine-modified and OKT3 pre-treated MLRs were set up with irradiated HLA fully- or partially-matched recipient PBMCs as stimulators, and live donor PBMCs as responders from 4 of the 6 individuals (Table 6.2). At 72 hours TCR Vβ sub-family distribution was measured in both CD8$^+$CD69$^+$ and CD8$^+$CD69$^-$ live responder cells.

Although criteria for defining size class peaks within TCR Vβ sub-families are well described after spectratyping based on measurement of CDR3 length by DNA PCR,[Peggs, Verfuerth, D'Sa, Yong, and Mackinnon 2003b] there is no published consensus on how overused or over-represented TCR Vβ sub-families measured by antibody/flow cytometric methods should be defined. A frequency above or below 3 standard deviations from the mean has been used to define over- and under-represented sub-families for resting values in individuals with immune defects.[Pierdominici *et al.* 2003] However, any definition of TCR Vβ overuse in cells responding to antigenic stimulation should take into account both relative change and absolute value of TCR Vβ frequency. Thus for the purposes of this analysis significantly over-expressed TCR Vβ sub-families in responder cells were defined as those with pre-stimulation frequencies within the normal range and post-stimulation frequencies which had increased relative to the pre-stimulation value by >/= 100% *and* to a level above the normal range (calculated from mean normal control values + (1.96 x standard deviation)). A diagram of the experimental schema is shown in Figure 6.1.

Pair	Responder HLA Type	Stimulator HLA type	CMV Peptide	CMV Dummy Peptide
1	A*0201 Class II not typed	n/a	NLV	None
2	A*0201;B*1501,3901; C*0303,1203; DRB1*0401,1101	n/a	NLV	TPR
3	A*0201,03001;B*39,27; C*01,02; DRB1*0301,0301	A B C DR matched	NLV	TPR
4	A*0201,*0101; B*08,*18; C*03,*07; DRB1* 0301	C locus mismatch	NLV	TPR
5	A*0201,*24;B*0702,*1401; C*07,*08; DRB1*0401,*0701	A, B, C, DR matched	NLV	DAN
6	A*0201,*31; B*44,*35; C*05,*12; DRB1*1501,*1401	A and C locus mismatch	NLV	DAN

NLV= NLVPMVATV TPR= TPRVTGGGAM DAN= DANDIYRIF

Table 6.2 HLA typing and CMV peptides used to assess TCR Vβ sub-family distribution after CMV peptide stimulation and HLA A, B, C and DR partially/fully matched allostimulation. n/a=not applicable.

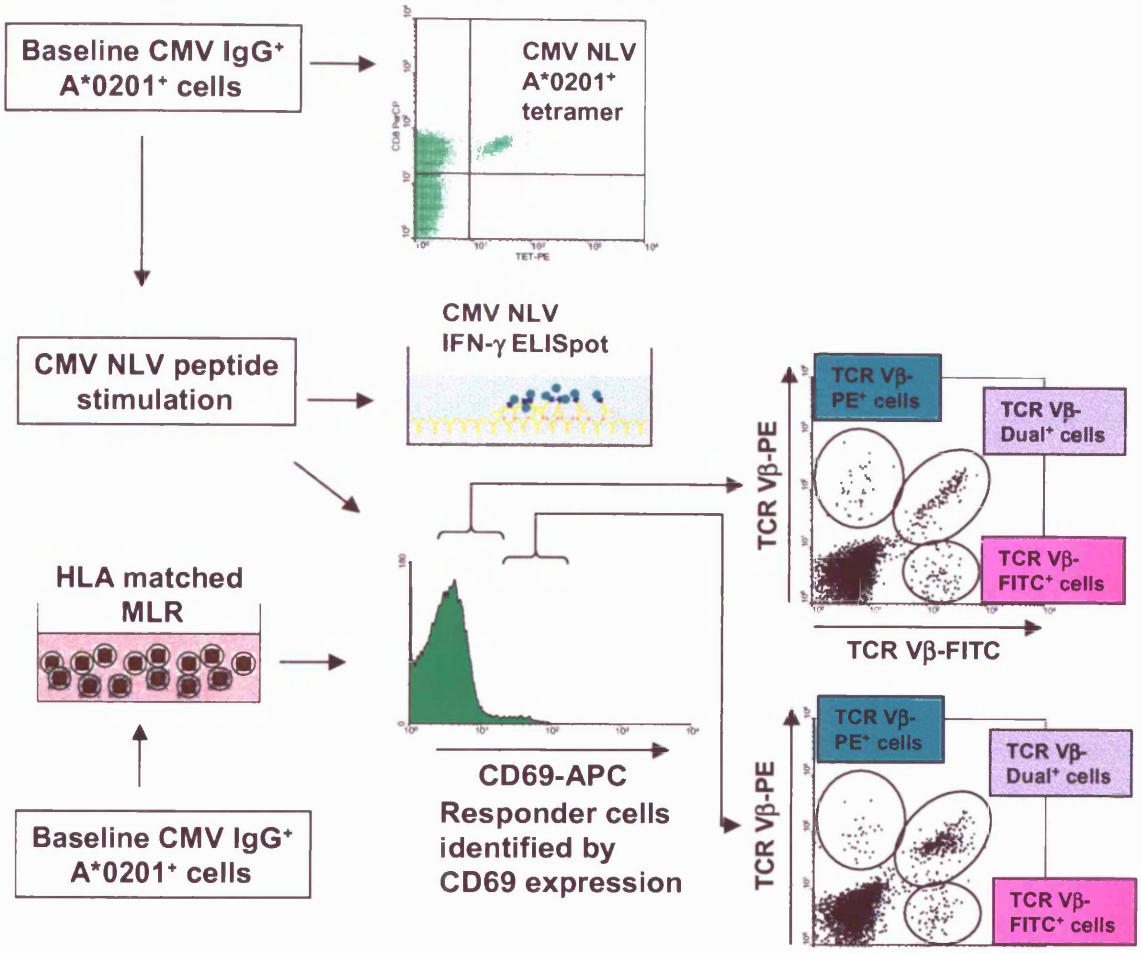

Figure 6.1 Schema for the experiments described in this chapter

6.4 Results

6.4.1 Optimisation of CMV peptide Stimulation Technique

CMV-specific CTLs were identified by the expression of CD69 after stimulation with NLV peptide.

Only CD69 antibodies conjugated to APC and PerCP were investigated, suitable for subsequent use in conjunction with the dual fluorochrome (PE/FITC) TCR Vβ antibody kit. APC-conjugated BD murine anti-CD69 antibody demonstrated the highest signal to noise ratio of those tested in preliminary experiments and was selected for use in further work (data not shown).

The dose of NLVPMVATV peptide required for optimal CD69 responses varied between individual HLA A*0201$^+$ CMV IgG$^+$ subjects. In 6 different peptide dose-titrations in 4 different healthy HLA A*0201$^+$ CMV IgG$^+$ individuals, the greatest mean CD3$^+$CD8$^+$CD69 response (1.5%+/-0.4%) was seen at a NLV peptide dose of 5μg/10^6 PBMCs with 12 hours stimulation. The CD69 response to NLV peptide stimulation was CD3$^+$CD8$^+$-specific with a CD3$^+$CD4$^+$ CD69 response of only 0.2%+/-0.1% (Figure 6.2).

A stimulatory peptide dose of 5μg/ 10^6 PBMCs was used for all subsequent experiments.

As CTL IFN-γ responses to peptide stimuli are well known to occur within 4-6 hours the CD69 response was assessed at 4 and 12 hours. No upregulation of expression of CD69 was seen after 4 hours of stimulation (data not shown) whereas CD69 expression was reliably seen by seen by 12 hours.

No additional benefit was seen when PBMCs were stimulated with peptide at a cell concentration of 5x10^6 /ml when compared to stimulation at 10^6 /ml.

For the sake of uniformity the latter concentration was used.

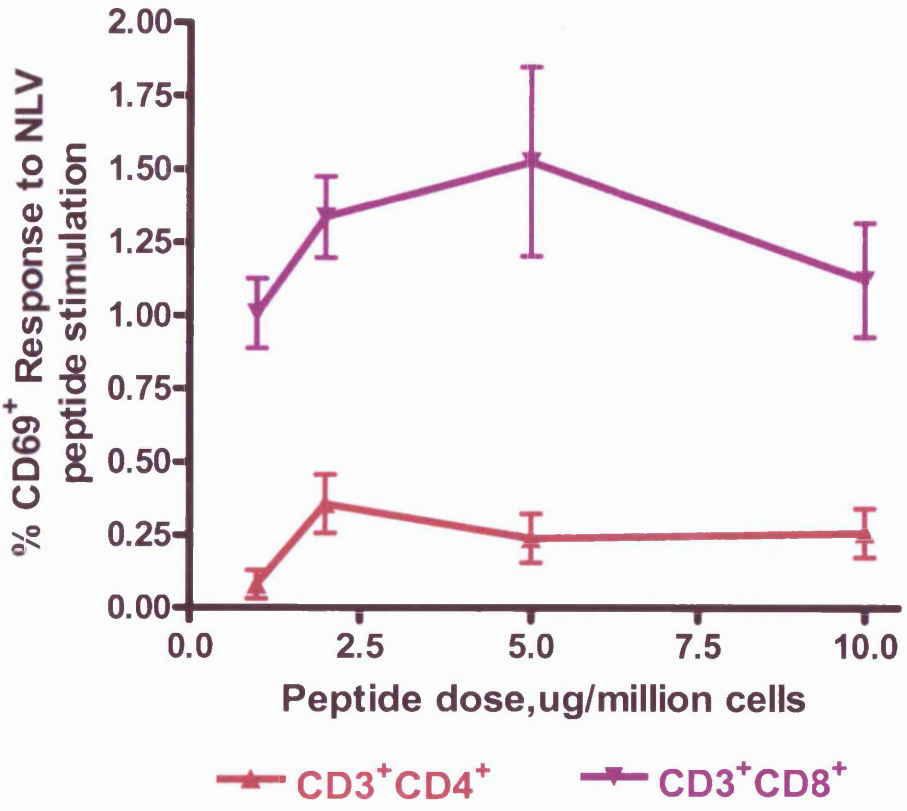

Figure 6.2 CD69 response to NLV peptide stimulation in HLA A*0201$^+$ CMV IgG$^+$ individuals: effect of peptide dose (results of 6 different peptide dose-titrations in 4 different healthy HLA A*0201$^+$ CMV$^+$ individuals). Error bars represent standard deviation.

6.4.2 CD69 Responses Specific to CMV peptide Stimulation

CD69 response specific to NLV peptide stimulation was defined as the percentage of cells positive for CD69 after NLV peptide stimulation - percentage of cells positive for CD69 after stimulation with dummy CMV peptide (i.e. CMV peptide restricted to an HLA Class I molecule not possessed by the cells being tested).

In the study of 16 HLA A*0201$^+$ CMV IgG$^+$ individuals the CD69 response specific to NLV peptide stimulation was 0.8% (+/-0.9%) of CD3$^+$ cells, 0.9%(+/-0.7%) of CD3$^+$CD8$^+$ cells and 0.3% (+/-0.4%) of CD3$^+$CD4$^+$ cells.

Thus the response to NLV peptide stimulation was relatively specific to CD3$^+$CD8$^+$ cells as was expected using a direct peptide stimulation protocol.

4 of the 16 HLA A*0201$^+$ individuals tested were also B*0702$^+$. Three of these individuals demonstrated CD3$^+$CD8$^+$ CD69 responses specific to NLV peptide stimulation below and one above the median response (not significant in a sign rank test). One of these individuals had a much larger CD3$^+$CD8$^+$ CD69 response specific to the HLA B*0702-restricted CMV-peptide TPR than to NLV (5.3% and 0.79% respectively). This pattern of immunodominant response is well reported but not universal in HLA*0201$^+$B*0702$^+$ individuals.[Lacey *et al.* 2003]

6.4.3 NLV-HLA A*0201 tetramer$^+$ Cell Frequencies

In the same 16 HLA A*0201$^+$ CMV IgG$^+$ individuals the baseline frequency of NLV-HLA A*0201-tetramer$^+$ cells was 1.39% (+/-1.06%) of CD3$^+$CD8$^+$ cells. In one individual the NLV-HLA A*0201 tetramer frequency was enriched by culture with autologous monocyte-derived dendritic cells and whole CMV antigen using the protocol described by Peggs et al.[Peggs, Verfuerth, Pizzey, Ainsworth, Moss, and Mackinnon 2002] The frequency of NLV-HLA A*0201 tetramer$^+$ cells was increased from 1.30% to 5.92% of CD3$^+$CD8$^+$ cells after culture and the number of cells expressing CD69 in response to NLV peptide was correspondingly increased from 0.9% to 3.81% of CD3$^+$CD8$^+$ cells. In all individuals tested the CD69 response in CD3$^+$CD8$^+$ cells was lower than the frequency of baseline NLV-HLA A*0201 tetramer$^+$ CTLs (Figure 6.4). The mean

frequency of $CD69^+$ CTLs after NLV peptide stimulation was 65% +/- 23% of the frequency of NLV $tetramer^+$ cells (p=0.0002 in a two-tailed paired Student's t-test). The frequency of $CD69^+$ $CD3^+CD8^+$ cells following peptide stimulation was well correlated with the baseline frequency of NLV-HLA A*0201 $tetramer^+$ cells in these individuals. The effect of peptide stimulation on tetramer frequency could not be measured as the addition of exogenous stimulatory peptide prevented the subsequent binding of tetramers to CTLs (data not shown).
In the four $HLA*0201^+B*0702^+$ individuals NLV-HLA A*0201 $tetramer^+$ cell frequencies were ranked 4^{th}, 8^{th}, 11^{th} and 16^{th} of 16 individuals assessed.
CD69 responses were closely correlated with baseline NLV-HLA A*0201 tetramer frequency although in 15 of 16 individuals tested the baseline NLV-HLA A*0201 tetramer frequency was greater than the frequency of $CD3^+CD8^+$ $CD69^+$ cells after peptide stimulation (Figures 6.3 and 6.4(A)).

6.4.4 CMV peptide-stimulated IFN-γ ELISpot-Reactive Cell Frequencies

The frequency of CMV-specific $CD3^+CD8^+$ cells in NLV peptide-stimulated IFN-γ ELISpot assay (assessed in 10 of the 16 individuals) was 0.30%+/-0.12%.
The mean frequency of IFN-γ ELISpot reactive CTLs after NLV peptide stimulation was 31% +/- 22% of the frequency of NLV $tetramer^+$ cells and 48%+/-13% of the frequency of $CD69^+$ CTLs after NLV peptide stimulation (p=0.002 and p=0.02 respectively in a paired two-tailed Student's t test)
In the 3 $HLA*0201^+B*0702^+$ individuals tested NLV- peptide-stimulated IFN-γ ELISpot positive cell frequencies were ranked 5^{th}, 7^{th}, and 10^{th} of the 10 individuals assessed. $CD3^+CD8^+$ CD69 responses following NLV peptide stimulation were closely correlated to the frequency of CMV-specific $CD3^+CD8^+$ cells in the NLV peptide-stimulated IFN-γ ELISpot assay although in 8 of 10 individuals tested the CD69 response was greater than the ELISpot reactive cell frequency (Figures 6.3 and 6.4(B)).

6.4.5 CMV peptide-stimulated Intracellular IFN-γ Generation

In four individuals intracellular IFN-γ generation was determined after NLV peptide stimulation under the same conditions. Intracellular IFN-γ generation

was seen in 0.91%+/-0.69% of $CD3^+CD8^+$ cells. In all individuals the frequencies of NLV peptide-stimulated $CD3^+CD8^+$ IFN-γ generating cells were 55.9%+/-21% of the frequency of $CD3^+CD8^+$NLV-HLA A*0201 tetramer$^+$cells (p=0.07 in a two-tailed paired Student's t test), 86%+/- 26% of the frequency of NLV peptide-stimulated $CD3^+CD8^+CD69^+$ cells (not significant) and 122% +/- 78% of the frequency of $CD3^+CD8^+$ NLV-HLA A*0201 IFN-γ ELISpot positive cells (not significant).

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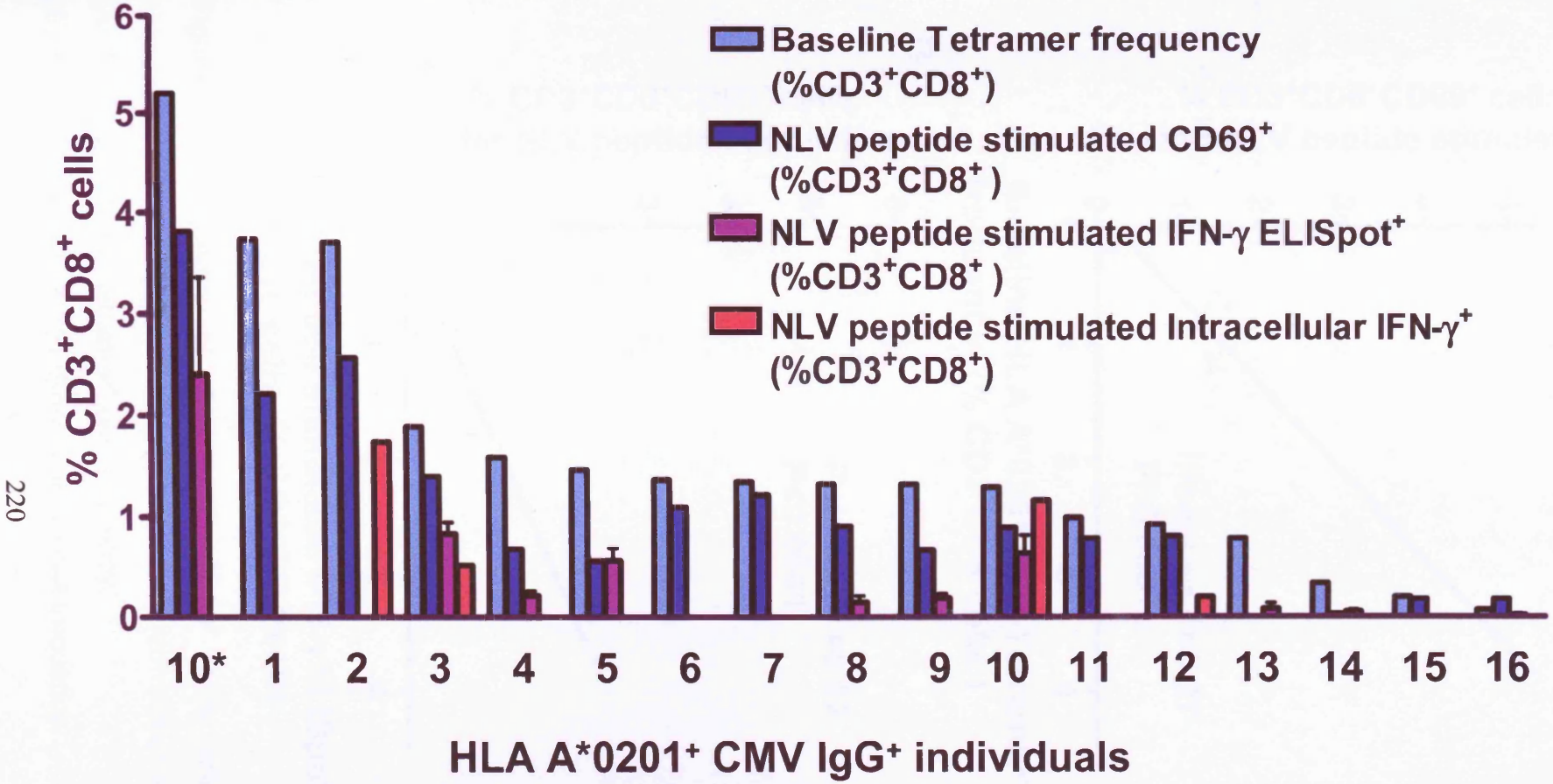

Figure 6.3 Frequency of NLV-HLA A*0201 tetramer$^+$ cells, CD3$^+$CD8$^+$ CD69$^+$ cells, IFN-γ ELISpot reactive cells and intracellular IFN-γ$^+$ cells after NLV peptide stimulation in HLA A*0201$^+$ CMV IgG$^+$ healthy individuals. 10* represents values obtained after CMV antigen-stimulated dendritic cell enriched cell culture of cells from individual 10. Individuals 4, 8, 11 and 16 were also HLA B*0702$^+$.

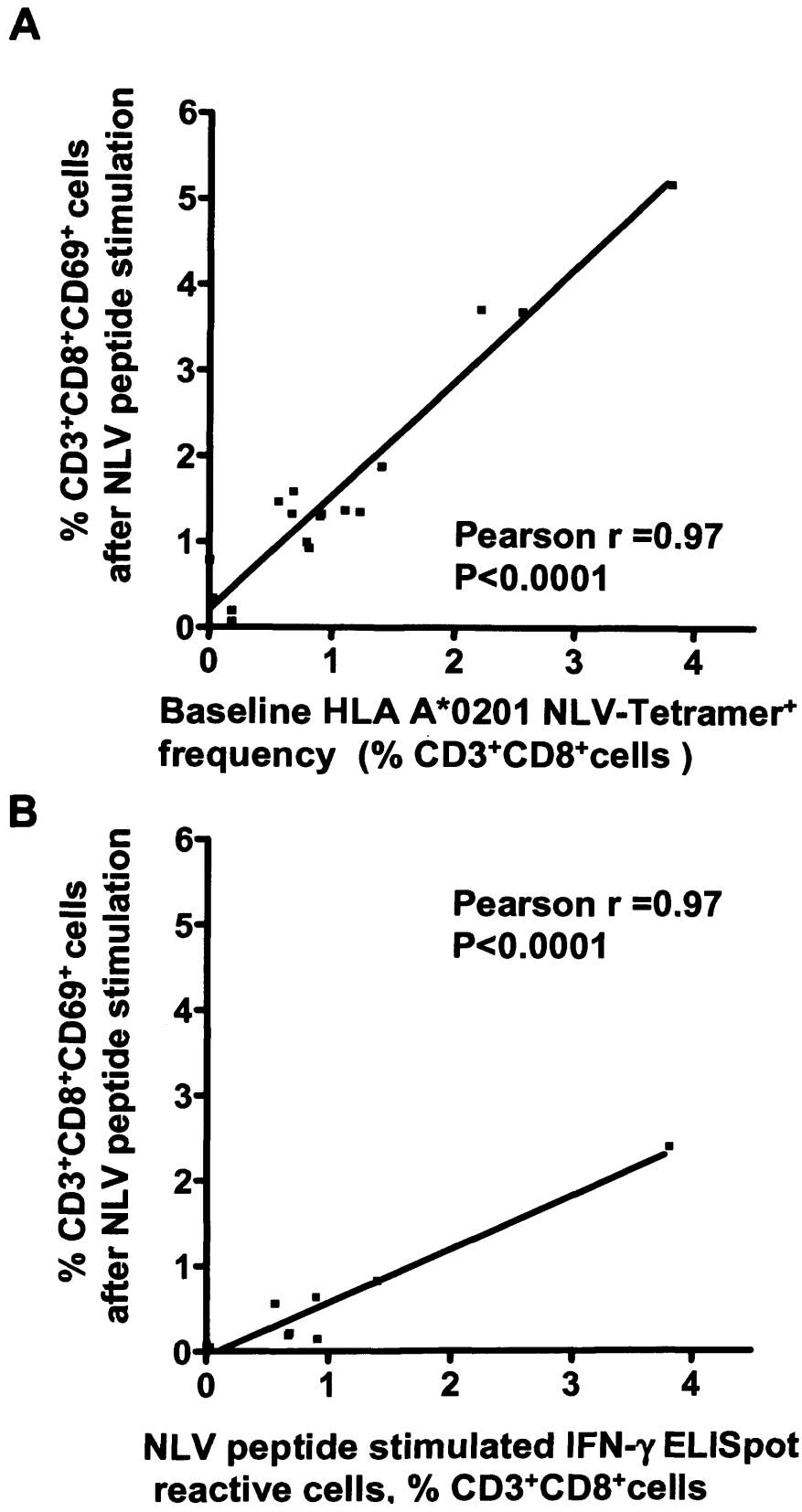

Figure 6.4 Frequency of $CD3^+CD8^+$ $CD69^+$ cells after NLV peptide stimulation in HLA $A*0201^+$ CMV IgG^+ individuals: correlation with;

(A). NLV-HLA A*0201 $tetramer^+$ cell frequency.

(B). NLV stimulated IFN-γ ELISpot-reactive cell frequency.

6.4.6 TCR Vβ Sub-family Distribution in CMV Peptide Responder Cells

The TCR Vβ sub-family distribution of $CD8^+$ cells was determined on 12 healthy subjects and normal ranges generated (as mean +/- 1.96 x (standard deviation)).The mean frequencies of individual TCR Vβ sub-families determined by direct staining of PBMCs (as opposed to whole blood) and without additional CD3 antibody staining were closely correlated with the normal ranges supplied by the manufacturers. PBMC stained $CD8^+$ TCR Vβ sub-family frequencies were closely correlated to the manufacturer's normal values (Pearson co-efficient of correlation of 0.93, $p<0.0001$, and linear regression graph slope of 1.18 +/-0.08 indicating that the PBMC method gave a generally slightly higher value for TCR Vβ sub-family frequencies than the whole blood staining method). TCR Vβ sub-family frequencies in $CD4^+$ cells were also derived from the non-$CD8^+$ cell fraction after direct staining of PBMCs. TCR Vβ sub-family frequencies for $CD4^+$ cells derived thus were also closely correlated to the manufacturer's normal values for $CD3^+CD4^+$ cells (Pearson co-efficient of correlation of 0.97, $p<0.001$), with a linear regression slope of 0.63+/-0.03 indicating that the PBMC non-$CD8^+$ method gave a generally lower value for frequencies of TCR Vβ sub-families than the whole blood $CD3^+CD4^+$ method. This was expected as the non-$CD8^+$ cell fraction also contains B cells and $CD8^-$ and $CD8^{weak}$ NK cells in addition to $CD4^+$ cells. This finding emphasises the importance of establishing and using the normal ranges for the specific method and antibodies used.

TCR Vβ sub-family distribution was assessed in 6 HLA $A*0201^+$CMV IgG^+ individuals in baseline (unmanipulated) cells and in $CD8^+CD69^+$ cells and $CD8^+CD69^-$ cells following stimulation with NLV peptide.

Following NLV peptide stimulation the TCR Vβ sub-family distribution in $CD8^+$ $CD69^-$ non-responders closely resembled that of baseline unmanipulated $CD8^+$ cells in all individuals. In contrast, the TCR Vβ sub-family distribution in $CD8^+CD69^+$ responders demonstrated significant skewing with an increase in the frequency of some TCR Vβ sub-families and a decrease in others within each individual. The mean percentage modular perturbation (either increase or decrease) from the baseline value for TCR Vβ sub-family frequency in $CD8^+$ cells was 69% +/- 27% for $CD69^+$ responders and only 17% +/- 4% for $CD69^-$

cells (p=0.005 in an unpaired two-tailed Student's t-test, with Welch's correction).

The values for TCR Vβ sub-family frequencies in non-$CD8^+CD69^-$ cells closely resembled that of baseline unmanipulated non-$CD8^+$ cells in all individuals. The mean modular perturbation from the baseline value for TCR Vβ sub-family frequency in non-$CD8^+$ cells was not significantly greater in $CD69^+$ cells (26% +/- 6.4%) than for $CD69^-$ cells (15% +/- 3.2%) in an unpaired two-tailed Student t test, with Welch's correction.

Although all 6 individuals tested demonstrated marked increases in some TCR Vβ sub-family frequencies in $CD8^+CD69^+$ cells above baseline values, overused TCR Vβ sub-families (as defined above) after NLV peptide stimulation were identified in only 4 of the 6 individuals tested (Figure 6.5). The median number of overused TCR Vβ sub-families in these individuals was 3. Only one individual tested had one selectively overused individual TCR Vβ sub-family in non-$CD8^+$ $CD69^+$ cells whereas all other individuals had no individual selectively overused TCR Vβ sub-families in non-$CD8^+$ $CD69^+$ cells.

In the 4 individuals who demonstrated individual overuse of TCR Vβ sub-families in $CD8^+$ $CD69^+$ cells after NLV peptide stimulation, one overused TCR Vβ 13.6, (individual 2), one overused TCR Vβ 5.2 (individual 4) one TCR Vβs 4.0, 7.1, 11.0 and 13.6 (Individual 5) and one TCR Vβs 2.0, 11.0 and 23.0 (individual 6). These data are shown in Figure 6.7, along with published data from three related studies for comparison.

TCR Vβ sub-family distribution in $CD8^+$ cells was also assessed in individual 2 following NLV peptide stimulation of cells which had been subjected to 3 rounds of stimulation with whole CMV antigen over 21 days with autologous monocyte-derived dendritic cell enrichment. TCR Vβ sub-family 13.6 was overused (in common with that seen after NLV peptide stimulation of fresh PBMCs in this individual) and also TCR Vβ 18.0 (which was not overused in fresh PBMCs from this individual stimulated with NLV, Figure 6.7).

Of the two other individuals tested one did show some evidence of selective TCR Vβ overuse with increases of >/=100% above baseline in $CD8^+CD69^+$ cells (in TCR Vβ sub-families 4.0 and 18, and 7.2 respectively) following PBMC stimulation with NLV peptide but the absolute values did not increase to levels above the normal range.

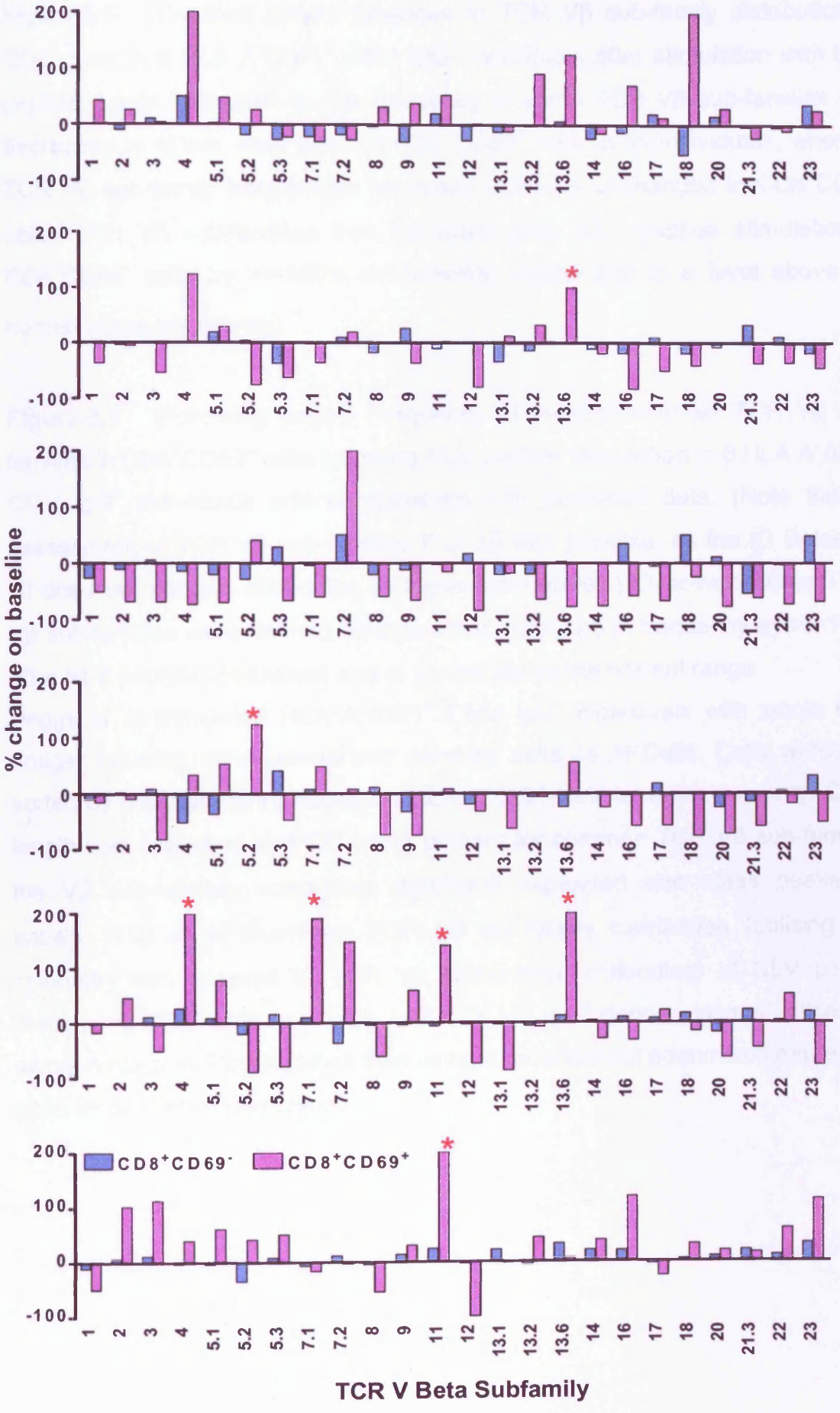

Figure 6.5 (See over for figure legend).

Figure 6.5 (Previous page). Changes in TCR Vβ sub-family distribution in $CD8^+$ cells in 6 HLA $A*0201^+$ CMV IgG^+ individuals after stimulation with NLV peptide. Large increases in the frequency of some TCR Vβ sub-families with decreases in others were seen in $CD8^+CD69^+$ cells in all individuals, whereas TCR Vβ sub-family frequencies remained relatively unchanged in $CD8^+CD69^-$ cells. TCR Vβ sub-families that increased after NLV peptide stimulation in $CD8^+CD69^+$ cells by >/=100% on baseline values and to a level above the normal range are marked *.

Figure 6.6 (Following page). Frequency of over-represented TCR Vβ sub-families in $CD8^+CD69^+$ cells following NLV peptide stimulation in 6 HLA $A*0201^+$ CMV IgG^+ individuals and comparisons with published data. (Note that no assessment of TCR Vβ sub-families 6 or 15 was possible, as the IO Betamark kit does not contain antibodies to these sub-families.) Over-represented TCR Vβ sub-families were defined as those that increased in frequency by >/=100% after NLV peptide stimulation *and* to values above the normal range.
Peggs et al stimulated HLA $A*0201^+$ CMV IgG^+ individuals with whole CMV antigen utilising monocyte-derived dendritic cells as APCells. Cells were then sorted by flow cytometry based on HLA A*0201-NLV tetramer positivity. CDR3 length was assessed by PCR using primers for common TCR Vβ sub-families: the Vβ sub-families containing significant expanded size class peaks are shown. Wills et al examined TCR Vβ sub-family distribution (utilising flow cytometry with a panel of TCR Vβ monoclonal antibodies) of NLV peptide responding $CD8^+$ cells in 4 HLA $A0201^+$ CMV IgG^+ donors. Hamel utilised the same strategy as Peggs (other than using a recombinant adenovirus expressing pp65 for antigen presentation).

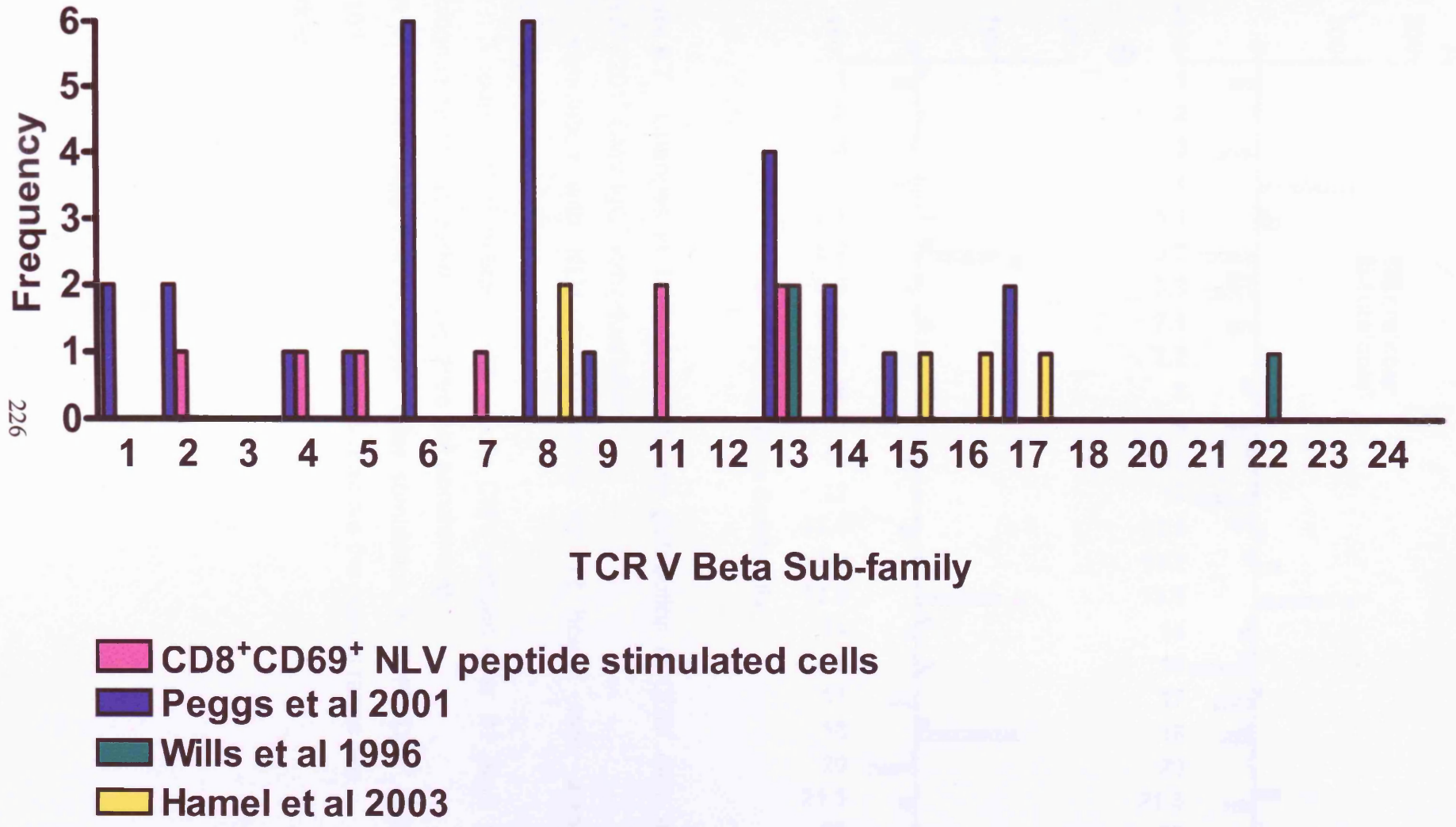

Figure 6.6 (See above for figure legend)

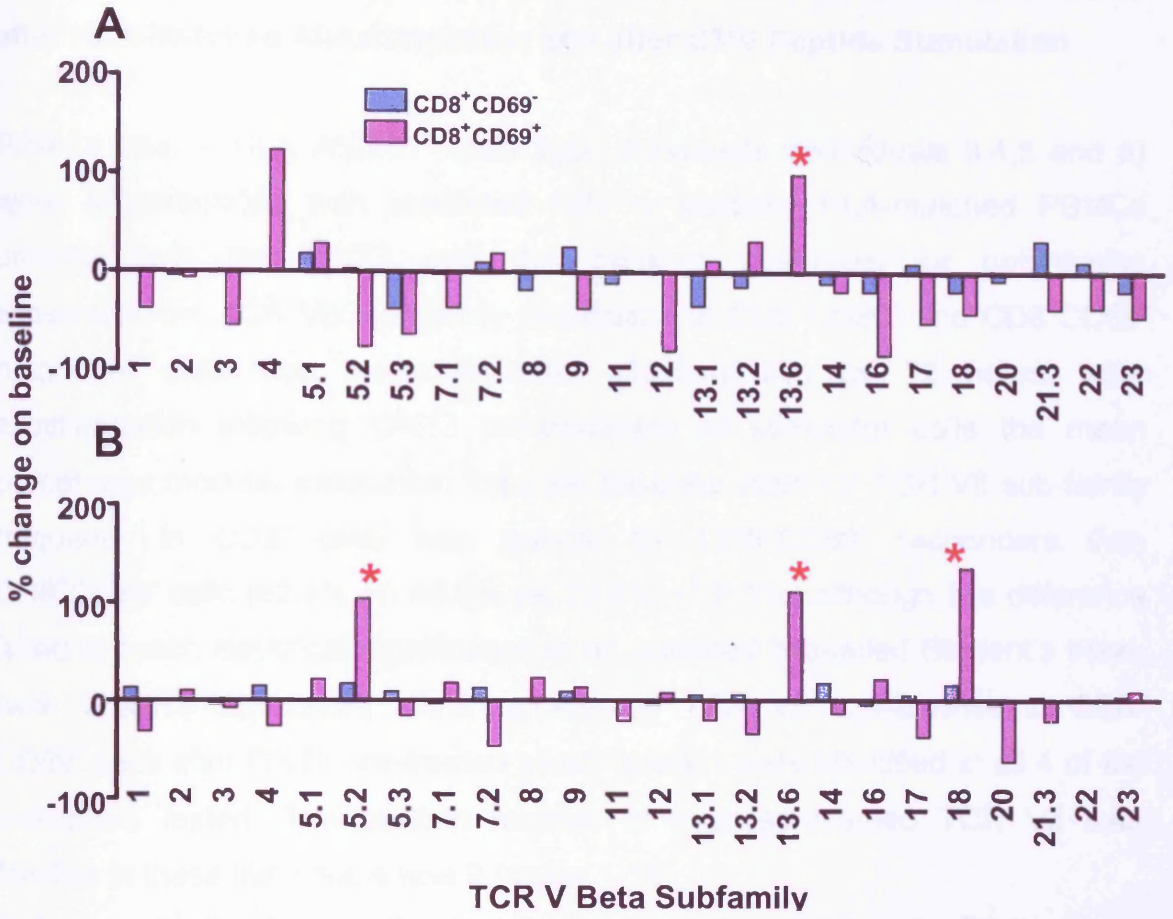

Figure 6.7 Changes in TCR Vβ sub-family distribution in $CD8^+$ cells in an HLA $A*0201^+$ CMV IgG^+ individual after;

(A). stimulation with NLV peptide alone for 12 hours under standard conditions

(B). 3 rounds of stimulation with whole CMV antigen over 21 days (with autologous monocyte-derived dendritic cell enrichment).

TCR Vβ sub-families that increased after stimulation in $CD8^+CD69^+$ cells by >/=100% on baseline values, and to a level above the normal range are marked *.

6.4.7 Comparison of TCR Vβ Sub-family Distribution within Individuals after HLA-Matched Allostimulation and after CMV Peptide Stimulation

PBMCs from 4 HLA A*0201$^+$ CMV IgG$^+$ individuals (Individuals 3,4,5 and 6) were allostimulated with irradiated fully or partially HLA-matched PBMCs utilising both the OKT3 and the cytokine strategies for potentiating allostimulation. TCR Vβ sub-family distribution in CD8$^+$CD69$^+$ and CD8$^+$CD69$^-$ responder cells was measured after allostimulation for 72 hours. After allostimulation following OKT3 pre-treatment of stimulator cells the mean percentage modular petubation from the baseline value for TCR Vβ sub-family frequency in CD8$^+$ cells was greater for CD8$^+$CD69$^+$ responders than CD8$^+$CD69$^-$ cells (82.9% +/- 46.6% vs. 27.4% +/- 8.1%) although this difference failed to reach statistical significance in an unpaired two-tailed Student's t-test, (with Welch's correction). Over-represented TCR Vβ sub-families in CD8$^+$ CD69$^+$ cells after OKT3 pre-treated allostimulation were identified in all 4 of the individuals tested. The median number of over-represented TCR Vβ sub-families in these individuals was 2 (range 1-10).

Following allostimulation with cytokine pre-treated stimulators for 72 hours the mean percentage modular perturbation from the baseline value for TCR Vβ sub-family frequency in CD8$^+$ cells was significantly greater in CD8$^+$CD69$^+$ responders (79.8% +/- 9.0%) than in CD8$^+$CD69$^-$ cells (38.3% +/-17.0%) ($p=0.02$ in an unpaired two-tailed Student's t-test). Over-represented TCR Vβ sub-families in CD8$^+$CD69$^+$ cells after cytokine-modified MLR allostimulation were identified in all 4 individuals tested. The median number of over-represented TCR Vβ sub-families in these individuals was 2 (range 1-4). TCR Vβ sub-family overuse in CD8$^+$CD69$^+$ cells following HLA-matched allostimulation using OKT3 or cytokine techniques is summarised in Table 6.3. Evidence of shared preferential usage of TCR Vβ sub-families after allostimulation and after CMV-peptide stimulation was seen, with 3 of 4 pairs tested demonstrating over-representation of a common TCR Vβ sub-family after OKT3 pre-treated allostimulation and NLV peptide stimulation, whereas 1 of 3 pairs tested demonstrated over-representation of a common TCR Vβ sub-family after cytokine pre-treated allostimulation and NLV peptide stimulation.

Pair	HLA Matching	Allostimulation technique	Over-represented TCR Vβ sub-families in $CD8^+CD69^+$ cells
3	A B and DR fully matched	OKT3	1, 13.2, 21.3
		Cytokine	16
4	A, B and DR fully matched	OKT3	1, 2, 4, **5.2**, 5.3, 7.1 13.2, 13.6, 18, 22
		Cytokine	nd
5	A, B and DR matched	OKT3	**4**
		Cytokine	**4**, 5.1, 13.6, 22
6	Single A locus mismatch	OKT3	**11**
		Cytokine	9, 21.3

Table 6.3 Over-represented TCR Vβ sub-families in HLA fully or partially matched $CD8^+CD69^+$ alloresponder cells. TCR Vβ sub-families also over-represented in $CD8^+CD69^+$ cells following NLV peptide stimulation are marked in **red**. nd= not done

6.5 Chapter Discussion

These results demonstrate that CD69 can be used to identify HLA A*0201$^+$ CD8$^+$ T cells activated specifically by the addition of the HLA A*0201-restricted CMV peptide NLV. These responding CD8$^+$ cells were likely have been activated via their TCRs by the presentation of NLV peptide in the context of HLA A*0201, although proof that this was the case would be provided by demonstration that activation of CD8$^+$ cells could be blocked by the addition of anti-HLA Class I antibody. However the CD69$^+$ responses were restricted to CD8$^+$ cells and the frequency of CD8$^+$CD69$^+$ cells following NLV peptide stimulation was well correlated with the frequency of NLV-HLA A*0201 tetramer$^+$ cells.

The frequency of NLV-stimulated CD8$^+$CD69$^+$ cells was significantly lower than the frequency of NLV-HLA A*0201 tetramer$^+$ cells indicating that only a proportion of NLV-HLA A*0201 tetramer$^+$ cells would be identified by CD69 expression following single round of NLV peptide stimulation. However the frequency of NLV peptide-stimulated CD8$^+$CD69$^+$ cells was greater than the frequency of CD8$^+$IFN-γ ELISpot-reactive cells, suggesting that following NLV peptide stimulation CD69 expression is likely to identify functional (antigen-responding) CTLs, including (but not limited to) IFN-γ secreting cells. NLV peptide-stimulated CD69$^+$ cells may include perforin-secreting cells.

One round of NLV peptide stimulation resulted in upregulation of CD69 on a relatively small percentage of CD8$^+$ responder cells and repeated rounds of peptide stimulation would be needed to enable the selection of significant numbers of CMV-specific CTLs based on their expression of CD69. Moreover, the generation of CD4$^+$ CMV-specific cells, necessary to provide $T_H1/2$ activity needed to sustain antiviral activity in vivo, would require the use of whole CMV antigen and professional APCells.

Selective TCR Vβ sub-family usage in CTLs in HLA A*0201$^+$ individuals has been described after repeated CMV NLV peptide stimulation over 10-15 days with IL-2 supplementation, measured by flow cytometry utilising panels of antibodies directed against different TCR Vβ sub-families. [Weekes *et al.* 1999;Wills *et al.* 1996b] Selective expansions of TCR Vβ sub-families have latterly been demonstrated by TCR Vβ spectratyping by CDR3 gene fragment

PCR in NLV-HLA A*0201 tetramer$^+$ CTLs, after repeated administration of whole CMV antigen in dendritic cell enriched cultures or by recombinant adenovirus expressing human CMV pp65.[Hamel *et al.* 2003;Peggs, Verfuerth, *et al* 2003c]

There are no published data comparing the TCR Vβ sub-family usage in CMV reactive CTLs and alloreactive CTLs within individuals.

Selective overuse of TCR Vβ sub-families in NLV peptide-stimulated CD8$^+$CD69$^+$ cells was seen, although TCR Vβ sub-family distribution was not as extremely skewed as previously reported after repeated/prolonged NLV peptide stimulation.[Weekes *et al* 1999] The latter observation is probably a result of the limitations of a single round of peptide stimulation; T cells with affinity for HLA A*0201-NLV peptide are activated but there is limited time available for a high-affinity activated T cell clone to proliferate and achieve relative dominance within the T cell pool. Additionally no exogenous cytokines or co-stimulation (e.g. IL-2, anti-CD28) were used to augment antigen presentation or T cell activation. Early T cell responses to NLV peptide identified by CD69 expression may not be as focussed as T cell responses after repeated rounds of antigenic stimulation; this has implications for cell sorting techniques based on CD69 expression after CMV-peptide stimulation.

TCR Vβ sub-family distribution in CD8$^+$CD69$^+$ cells after NLV peptide stimulation varied between individuals but TCR Vβ sub-families 11 and 13.6 were over-represented in 2 of 6 individuals.

Weekes et al reported over-representation of TCR Vβ sub-family 13 in 2 of 3 HLA A*0201$^+$ individuals following repeated rounds of NLV peptide stimulation. Peggs found size class peaks expanded in Vβ 13 by CDR3 PCR in NLV-HLA A*0201 tetramer$^+$ cells following repeated rounds of stimulation with whole CMV antigen, and also in TCR Vβ sub-family 6 (the frequency of which was not determined by the panel of TCR Vβ sub-family epitope antibodies used here). Thus a single round of NLV peptide stimulation allows the identification of peptide-responding CD8$^+$ cells by virtue of their upregulated expression of CD69, with evidence of non-random TCR Vβ sub-family overuse consistent with that seen in the published literature after repeated rounds of antigenic stimulation.

In all 4 HLA fully/partially HLA-matched pairs, after 72 hours of allostimulation, responder $CD8^+CD69^+$ cells demonstrated skewing of TCR Vβ sub-family distribution (with 3 demonstrating individual over-represented TCR Vβ sub-families as defined in Chapter 6.3). Different TCR Vβ sub-families were over-represented within individuals after allostimulation using either the OKT3 technique or the cytokine technique.

In 3 pairs, responding $CD8^+$ cells demonstrated over-represented TCR Vβ sub-families in common after NLV peptide stimulation and OKT3 allostimulation, supporting the possibility of cross-reactivity of allospecific and CMV-specific CTLs which warrants further investigation. CDR3 PCR-based spectratyping of alloresponders identified by CD69 is not possible by virtue of the presence of contaminating DNA from allogeneic stimulator cells (which express CD69 post irradiation and upon apoptosis).[Chen *et al.* 1997] Confirmatory work using CDR3 PCR-based spectratyping could be done if a method to sort alloresponding cells from stimulators was developed. Further work could include crossover proliferative assays and cytotoxicity assays for alloreactivity in CMV responders (and the assessment of CMV-specific responses in alloresponding cells) to investigate whether alloreactive cells exhibit enhanced CMV reactivity and CMV reactive cells exhibit enhanced alloreactivity.

Assessment of donor T cell TCR Vβ sub-family distribution by flow cytometry could be used to produce more individually tailored cell fractions with depletion of specific TCR Vβ sub-families over-represented in donor cell fractions after allostimulation but not common to viral antigen-stimulated responses.

TCR Vβ sub-family distribution could also be used to differentiate donor T cells with specificity for tumour antigens from alloreactive T cells. [Jiang *et al.* 1997] In a recent murine model Vβ families exhibiting reactivity to the leukaemia cells were then enriched for and administered in an AHSCT model to assess *in vivo* GvL potential. In syngeneic transplants, enrichment for pools of selected Vβ families of T cells conveyed a beneficial GvL response to recipients. Furthermore, in a haploidentical allogeneic model, enrichment of myeloid leukaemia-specific TCR Vβ cells exhibited significant GvL responses with concomitant minimization of GvHD development. [Patterson and Korngold 2001] However a problem with these strategies is likely to arise as a result of the degree of degeneracy of TCR-MHC/peptide antigen interaction, and the

likely lack of sensitivity and specificity of any given TCR Vβ sub-family for a given mHag or viral antigen. Thus selective depletion of an individual TCR Vβ sub-family over-represented in T cells specific for such antigens is unlikely to remove all T cells specific for this antigen as others will belong to other TCR Vβ sub-families.

Chapter 7 Development of the CD69-based Selective Allodepletion Strategy to a Clinical Scale

7.1 Introduction

The aim of development of the strategy of CD69-based selective allodepletion is to apply the technique at a clinical level in order to reduce GvHD whilst improving immune reconstitution (and potentially preserving the GvL effect of donor allogeneic cells). Moreover in order to produce an allodepleted product of clinical relevance, development of the CD69 allostimulation and subsequent allodepletion techniques at a much larger scale was required.

To date selective allodepletion has been performed at a clinical scale using only the CD25-based strategy. André-Schmutz et al reported a phase 1/2 study of re-infusion of CD25-immunotoxin-mediated allodepleted T cells to HLA-mismatched paediatric recipients after AHSCT. To prepare selectively allodepleted T cells, 5×10^7 donor PBMCs were cultured *in vitro* in a one-way MLR with 5×10^7 irradiated (30 Gy) stimulating PBMCs. After 3 days of *in vitro* activation, the MLR culture was treated overnight at 37 C with 10^{-8} mol/L of anti-CD25 antibody-conjugated immunotoxin RFT5-SMPT-dgA in RPMI-1640 containing 20 mmol/L ammonium chloride (pH 7·8), to deplete $CD25^+$ alloreactive cells. After assessment of reduction of $CD25^+$ cells in after immunotoxin treatment (by flow cytometry) and confirmation of reduction of first party proliferative responses (less than 1% residual anti-host alloreactivity was recorded in 12 of 16 procedures) allodepleted cells were infused to the patient in escalating doses (1, 4, 6, and 8×10^5 T cells/kg). [Andre-Schmutz *et al* 2002].

Soloman et al have published details of a clinical-scale strategy of selective allodepletion of donor T cells, also based on immunotoxin-mediated destruction of $CD25^+$ alloreactive cells. [Solomon *et al* 2002] To prepare stimulator cells, healthy donors underwent leucopheresis (10-12 Litres of blood volume) and PBMCs were obtained by processing on the Fenwall CS300 Plus blood separator. Responder cells were generated from G-CSF primed healthy donors (10μg/kg/day for 5-6 days) who were then leucopheresed. Stimulator cells were cultured in X VIVO-15 with 5% autologous plasma and 100IU/ml recombinant IL-2 in 300, 1000 and/or 3000 ml Lifecell gas-permeable bags. Stimulator cells were suspended at 10^8/ml in culture medium and OKT3 (15μg/10^8 cells) for 20

minutes. $CD3^+$ cells were then isolated using sheep anti-mouse (SAM) magnetic beads and the MaxSep system. The stimulator cells were left in culture medium overnight to allow spontaneous detachment of the SAM magnetic beads. Stimulator $CD3^+$ cells were then expanded in 3000ml Lifecell bags pre-coated with OKT3 ($50\mu g/10^8$ cells) at a concentration of 0.6×10^6 cells/ml. Cells were expanded over 48-72 hours in this manner and then washed prior to cryopreservation. Responder T cells were obtained from the $CD34^-$ fraction after CD34 selection on the Isolex 300i automated device. Irradiated stimulator cells (2500 cGy) at a concentration of 5×10^6/ml were added to live responder cells at a S;R ratio of 1:1 and the MLR cultured under standard conditions for 72 hours. The RFT5-SMPT-dgA anti-CD25 immunotoxin was added at 24 and at 48 hours with ammonium chloride. Efficiency of depletion was assessed by flow cytometry and by proliferation assays with first party stimulators before and after selective allodepletion.

Using this strategy, stimulator cell selection and expansion yielded $>2 \times 10^{10}$ $CD3^+$ stimulator cells. After anti-CD25 immunotoxin mediated-selective depletion, recovery of cells was 70% and viability 85%, permitting a potential selectively allodepleted T cell dose of $> 10^8$ $CD3^+$ cells/kg recipient body weight. Proliferative responses to original stimulators after selective allodepletion were 2.6% of those seen with unmanipulated responders, with preservation of third party responses. This strategy has now been used for the preparation of selectively allodepleted T cells from HLA-matched sibling donors and the preliminary results of a Phase 1 safety study have been reported in abstract form.[Solomon *et al* 2003]

Recently, new and stringent regulations governing both the conduct of clinical trials and the environment in which cellular products for clinical use are produced and stored have been introduced. The European Clinical Trials Directive (EuCTD, 2001/20/EC ([Anon 2001]) was introduced in May 2004. The aims of this directive were to harmonise and simplify the administrative procedures and standards of protection for clinical trial participants. It has far-reaching consequences for the conduct of clinical trials, including those utilising the manufacture and *ex vivo* manipulation of cellular products. The EuCTD has been implemented in the UK through new national legislation drawn up for parliamentary approval by the Medicines and Healthcare Products Regulatory Authority (MHRA).

The environment within which a cellular product designed for human use is manipulated (and stored) has come under increasing scrutiny and regulation in recent years. The Foundation for the Accreditation of Cellular Therapy (FACT) has implemented programs of voluntary inspection and accreditation for laboratories involved in haematopoietic cellular therapy. These programs were based on the standards of the clinical and laboratory professionals of the American Society of Blood and Marrow Transplantation (ASBMT) and the International Society for Cellular Therapy (ISCT). FACT has collaborated with European colleagues in the development of the Joint Accreditation Committee in Europe (JACIE).

National guidelines have been periodically issued in the UK to address the manufacture and storage of cellular products (other than red cells, platelets and plasma products manufactured by the blood transfusion service). In 1997 the Department of Health (DoH) issued Guidance Notes on the Processing, Storage and Issue of Bone Marrow and Blood Stem Cells',[Department of Health 1997] which have been superseded by the Tissue Banking Code of Practice issued in 2001.[Department of Health 2001] The latter document covers all human tissues used for any therapeutic purpose (including clinical trials) including haematopoietic stem cells and donor leucocytes. This code of practice states that all products must be produced using good manufacturing practice (GMP) and under conditions of good laboratory practice (GLP) including microbiological screening of donors, bacterial testing of processed products, and storage of products in liquid nitrogen vapour. Furthermore GMP demands the use of validated procedures and processes with product definition, vigorous sterility testing, and records of reagents used. GLP requires the use of an accredited production laboratory utilising standard operating procedures (SOPs), Conformité Européene (CE)-marked and validated equipment, "closed" systems where possible, quality assurance and meticulous record keeping. This Tissue Banking Code of Practice is to be reviewed no later than June 2005.

Accreditation of laboratories covering all aspects of the laboratory environment, staff training, product manufacture, storage, release criteria and quality control is now awarded by the MHRA (formerly by the Medicines Control Agency, (MCA) up to 2003) 'on behalf of the DoH' in UK to tissue banks.

The development of the CD69-based allodepletion strategy to a clinical scale encompassed the production and testing of an antibody suitable for clinical *ex*

vivo use. Different monoclonal antibodies to different epitopes of CD69 have been described,[Gerosa *et al.* 1991] and thus depletion efficiency using a given cell sorting technique might be different using different anti-human CD69 antibodies. Several different techniques are available at a bench level for the immunomagnetic depletion of $CD69^+$ cells and some of these can be used in automated or semi-automated devices specifically designed and used for sorting allogeneic haematopoietic stem cell grafts prior to clinical use.

7.2 Aims of Experiments described in this Chapter

1. Identification of a CD69 antibody suitable for production (qualitatively and quantitatively) for use at a clinical scale;
2. Development of cell culture/allostimulation conditions at a clinical scale;
3. Testing of various technologies for CD69 expression-based depletion of alloreactive cells;
4. Development of depletion strategies quantitatively and qualitatively to a clinical scale.

7.3 Materials and Methods

7.3.1 Anti-human CD69 Antibody TP1 55.3

The monoclonal murine IgG2b anti-human CD69 antibody TP1 55.3[Cebrian *et al.* 1988] was produced in a culture fermentation system from murine hybridoma cells (Prof Sanchez-Madrid, University of Madrid, Spain). P3-X63-AG.8653 x BALB/c murine hybridoma cells were cultured in complete medium (with 10% FCS from transmissible spongiform encephalopathy (TSE) -free sources). Supernatant was centrifuged to remove cells and debris, filtered (200μm) and stored at -70°C.

The CD69 antigen is a 60 kDa disulfide-linked dimeric structure containing two differentially glycosylated forms (33 and 27 kDa) of a single core protein, which are covalently associated. TP1 55.3 antibody immunoprecipitates the 60 kDa homodimer and both 27 kDa and 33 kDa subunits from activated peripheral blood lymphocytes.[Cebrian *et al* 1988]

7.3.2 Anti-human CD69 Antibody-CH11

The monoclonal murine IgG1 anti-human CD69 antibody CH11 (Novocastra Laboratories, Newcastle upon Tyne, UK) was produced using a prokaryotic recombinant protein corresponding to the C terminal of the human CD69 molecule as an immunogen with the hybridoma partner mouse myeloma cell line (p3-NS1-Ag4-1) and was supplied directly from the manufacturers in solution at a concentration of 5μg/ml.

7.3.3 Bulk Allostimulation Strategies

Allostimulation using HLA-mismatched irradiated (30Gy) stimulator and live responder PBMCs was assessed by responder CD69 upregulation (Chapter 2.2.1-2) at 72 hours and proliferation (thymidine uptake) at 120 hours (Chapter 2.3.2) in various culture conditions as detailed in Table 7.1. The S:R ratio was constant at 1:1 and cells were cultured under standard conditions in complete medium with 10% human AB serum. For small-scale allostimulation

experiments fresh PBMCs were obtained from healthy volunteers. For large-scale HLA-mismatched allostimulation experiments, single-donor buffy coats were supplied from North London Transfusion centre and PBMCs were extracted by density gradient centrifugation (Chapter 2.1.3).

Resuscitated cryopreserved PBMCs from HLA-matched unrelated volunteer donors and AHSCT recipients were used to assess HLA-matched cytokine-modified allostimulation at an intermediate scale. HLA-matched stimulators were pre-treated with 1000iu/million cells of IFN-γ and TNF-α prior to use in the modified MLR, and IL-4 added to HLA-matched MLR cultures as described in Chapter 2.3.3. (The HLA typing of matched pairs used in larger scale allostimulation experiments is detailed in Table 7.2).

HLA Matching	Allostimulation Condition	Final Volume Of MLR	Responder Cells	
			Final concentration	Final number
HLA-Mismatched	96-well plate	*200μL*	*0.5 x 10^6/ml*	*10^5*
	24-well plate	*2mls*	*0.5 x 10^6/ml*	*10^6*
	160cm^3 Stericell bag	*140mls*	*1.5 x 10^6/ml*	*210 x 10^6*
HLA-Matched	96-well plate	*200μL*	*0.5 x 10^6/ml*	*10^5*
	24-well plate	*2mls*	*0.5 x 10^6/ml*	*10^6*
	160cm^3 Stericell bag	*100mls*	*0.5 x 10^6/ml*	*50 x 10^6*

Table 7.1 HLA-mismatched and HLA-matched allostimulation conditions for scale-up experiments.

7.3.4 Generation of CD69$^+$ T cells with Mitogens

In order to produce large number of CD69$^+$ T cells to evaluate cell-sorting systems, fresh human PBMCs were activated with the polyclonal mitogens *Phaseolus vulgaris* agglutinin (phytohaemagglutinin, PHA) or the combination of Pokeweed Mitogen (PMA) and Calcium Ionophore (CAI, all Sigma-Aldrich).

Phaseolus vulgaris agglutinin consists of two molecular species, an erythroagglutinin which has low mitogenic activity and high erythroagglutinin activity, and leucoagglutinin, which has high mitogenic and leucoagglutinating activity, but very low erythroagglutinating activity. PHA was supplied as a lyophilised powder and diluted in DMSO to a working concentration of 100ug/ml. 2μg of PHA was added to 1 ml of PBMCs (at a cell concentration of 10^6/ml) and the cell suspension incubated under standard conditions for 4-12 hours. PMA was supplied as a dry powder stored desiccated below -20°C. A stock dilution of PMA was prepared by dissolving PMA powder in DMSO to a concentration of 0.5mg/ml and immediately diluting 1:100 in HBSS. 20μL aliquots of stock dilution were stored at -20°C at a concentration of 5μg/ml. At the time of use aliquots were thawed and diluted 1:10 in complete medium to give a final working concentration of 500ng/ml. CAI was supplied as a dried powder stored desiccated below -20°C. A stock dilution was prepared by dissolution in DMSO to a concentration of 0.5mg/ml and stored at room temperature. A working concentration of 5μg/ml was prepared by diluting the stock 1:100 in complete medium. To activate fresh PBMCs with PMA and CAI, 50μL of working concentration of PMA and 100μL of working concentration of CAI were added to each 0.5mls of PBMC suspension (at a cell concentration of 1 x 10^6/ml) which were then incubated overnight under standard conditions. Following activation with mitogens, PBMC suspensions were centrifuged for 10 minutes at 200g and resuspended (at 10^6 cells/ml) twice. The proportion of T cells activated by mitogens was assessed by flow cytometry after staining with 5μL of CD3-FITC and CD69-PE /10^5cells, (both BD) (Chapter 2.2 1). Activated PBMCs were mixed with resting PBMCs to adjust the frequency of $CD3^+CD69^+$ cells to between 5-20% for subsequent cell sorting experiments.

7.3.5 Small Scale Eligix Microparticle Depletion

Depletion of 2 x 10^6 PBMCs containing 5-10% mitogen-activated $CD3^+CD69^+$ cells with TP1 55.3 antibody and the Eligix microparticle system (Chapter 2.5.3) was undertaken in three separate experiments with varying quantity of antibody used per million cells.

7.3.6 Small Scale Dynabead Depletion

The Dynabead immunomagnetic sorting system (Chapter 2.5.2) was tested with the TP1 55.3 antibody at a small scale. Depletion of 2 x 10^6 PBMCs containing 5-10% mitogen-activated $CD3^+CD69^+$ cells was performed with varying amounts of TP1 55.3 antibody and different Dynabead:cell ratios.

7.3.7 Intermediate Scale Dynabead Depletion

The CH11 murine IgG1 anti-human CD69 was tested with the Dynabead system at an intermediate scale using 5 HLA-matched unrelated donor-recipient pairs. The cytokine-modified MLR (Chapter 2.3.3) was used to potentiate allostimulation. 50 x 10^6 live responders were incubated with 50 x 10^6 irradiated stimulator cells in Lifecell 160cm^3 bags. Stimulators were pre-treated with IFN-γ and TNF-α and IL-4 added to the MLR culture as per the cytokine-modified MLR protocol (Chapter 2.3.3).

After initial optimizing experiments, 12.5µL (62.5 ng) /million responder PBMCs of CH11 antibody was used. Dynabeads were used at a bead: cell ratio of 5:1. Details of donor/recipient characteristics are shown in Table 7.2

CD69 expression on $CD3^+$ responder cells was determined by flow cytometry after 72 hours after co-culture with stimulators in the cytokine-modified MLR and following immunomagnetic depletion of $CD69^+$ cells. First and third party (HLA-mismatched) MLRs were set up with unmanipulated responder cells and allodepleted responder cells as described in Chapter 2.4.2.

Pair	Stimulator	Responder
1	A*0101, 2402 B*5101, 5201 C*1202, 0202 DRB1*1502,0701	HLA-matched at A, B, C and DR
2	A*0101, 02011 B*5701, 4002 C*0202, 0602 DRB1*1101,1104	HLA-matched at A, B, C and DR
3	A*02011, 3301 B*1402, 3501 C*0401, 0801 DRB1*	HLA-matched at A, B, C and DR
4	A*0201, 3101 B*1301, 1801 C*0601,0701 DRB1*1301,0701	HLA-matched at A, B, C and DR
5	A*0201, 2402 B*5101, 4402 C*0401, 0501 DRB1*1601,0407	HLA-matched at A, B, and DR Single C locus mismatch

Table 7.2 HLA typing of HLA-matched pairs used to evaluate allodepletion with the CH11 anti-human CD69 antibody and the Dynal Immunomagnetic cell sorting system at an intermediate scale.

7.3.8 Clinical Scale Dynabead Depletion

After initial small scale experiments to determine the efficiency of the Dynabead immunomagnetic cell sorting system, 3 clinical scale allostimulation and selective allodepletion procedures were performed using the Isolex 300i (with v2.5 software).

210 x 10^6 fresh stimulator PBMCs were obtained from single-donor buffy coats (Chapter 7.3.3) and suspended in complete medium with human AB serum at 3 x 10^6 cells/ml and irradiated. These were resuspended in a Lifecell 160cm^3 bag to which 70 mls of fresh live HLA-mismatched responder cells in complete medium with human AB serum (at 3 x 10^6 cells/ml) (from a single donor buffy coat) was added, to give a final responder concentration of 1.5 x 10^6 cells/ml. First and third party primary MLRs were set up in parallel. The bulk cell

suspension was incubated under standard conditions for 72 hours and CD69 expression on responder $CD3^+$ cells prior to and after depletion on the Isolex 300i automated device was measured by flow cytometry (Chapter 2.2.1-2). 12 mls (14.4μg) of TP1 55.3 was used (limited by the maximal volume allowable on the Isolex 300i) with a Dynabead:cell ratio of 5:1 for each procedure. A depletion procedure on the Isolex 300i involved following the automated path for positive selection up to a certain stage, and then recovery of the negative cell fraction was performed. Working buffer was made up as follows: 360mls of sodium citrate 4% solution (Baxter) and 120 mls of 25% Human Albumin Solution, (HAS, North London Blood Transfusion Centre) (or 150mls of 20% HAS) was added to a 3-litre bag of PBS (Baxter) under sterile conditions within a tissue culture hood. Total working buffer bag weight was greater than 3250g (and less than 7000g). 20mls of the working buffer was transferred into a sterile disposable 20ml syringe with a 21G needle and label 'Buffer Blank' (to be used as a substitute for releasing agent). 20mls of working buffer was reserved for Dynabead washing. 14.4 μg of TP1 55.3 CD69 antibody in a volume of 12 mls was drawn up into a syringe labelled 'CD69 antibody'.

The contents of one vial of Dynabeads were then removed (10 mls volume) and transferred to a sterile 50ml tube (Nunc) within a tissue culture hood.

10mls of working buffer was added to the tube to bring the total volume to 20mls and the tube was then exposed to the MPC-1 for 2 minutes. The supernatant was aspirated and discarded. The tube was then removed from the MPC-1 and the beads resuspended in 10 mls of working buffer and transferred to a sterile disposable 20ml syringe.

The Lifecell culture bag was then connected to the Isolex cell source bag (supplied with the Isolex disposable plastics kit) using a plasma transfer set (Baxter) and the cell suspension transferred from the Lifecell bag to the Cell Source bag. The plasma transfer set was sealed with 2 disposable plastic haemostats and a heat-sealer.

The Isolex 300i magnetic cell sorter was then powered up and the system initialisation step carried out. When the 'Select a procedure' screen appeared 'Positive Selection Only' was selected. The Isolex 300i disposable set was then installed as per manufacturers instructions. The buffer, buffer blank (dummy release agent), and CD69 antibody were then added sequentially to bags on

weight scale 6, 2, and 3 respectively. The Dynabeads were then added to the rocker arm chamber and the cell source bag connected to the C1 tube and attached to weight scale 1. Buffer was then transferred automatically via the disposable plastic tubing network to the release agent, antibody and cell source bags. After buffer transfer the platelet wash step was initialised. When the platelet wash was complete the contents of the CD69 antibody bag were pumped into the cell suspension. The antibody was then incubated with the cells for 15 minutes while the cell and antibody suspension recirculated through the spinning membrane to allow thorough mixing. When the antibody and cells finished incubation, the cells were then transferred to the rocker arm chamber for rosetting with the Dynabeads for 30minutes. At the end of this period, immediately prior to the magnetic separation step, the red STOP button was pressed to terminate the automated procedure. 'Cell Recovery Mode' was then entered, and option 4 ('Transfer chamber to Wash Bag 2') selected. After mixing for 2 minutes, magnetic separation of the cell suspension was activated and the cell suspension in the rocker arm chamber drained over the secondary magnet, into wash bag 2 on weight scale 5. After rinsing the drain path, cells were transferred from wash bag 2 to the end product bag on weight scale 4 and the red stop button pressed to end the depletion procedure. The end product bag containing the depleted cell suspension was heat sealed and removed.

The yield and viability of $CD3^{+}CD69^{-}$ cells was assessed by cell counting and trypan blue staining (Chapter 2 1.4) and the depletion efficiency of $CD3^{+}CD69^{+}$ cells by flow cytometry (Chapter 2.2.1-2). First and third party MLRs were set up using the depleted fraction as responders to assess the functional efficacy and specificity of allodepletion.

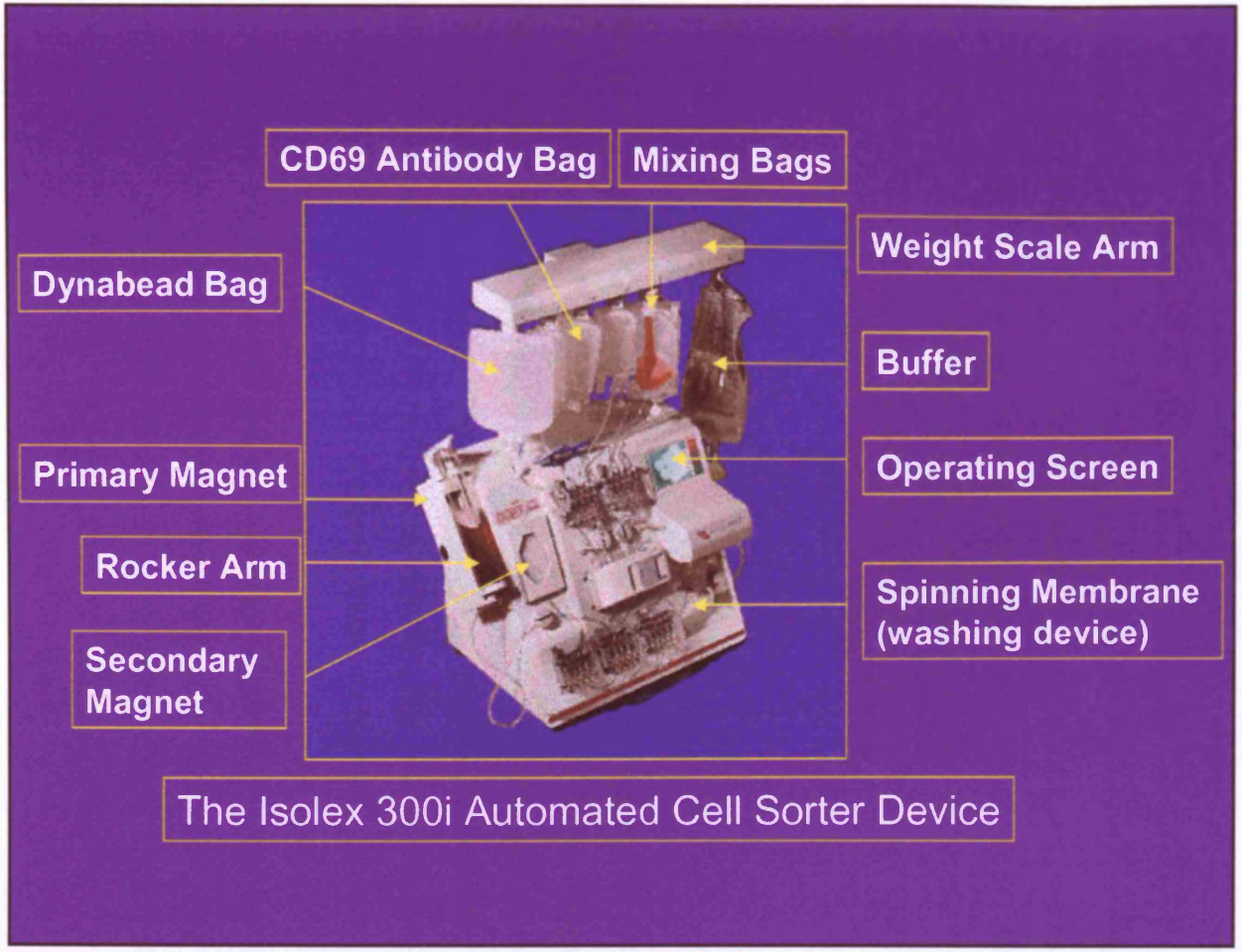

Figure 7.1 The Isolex 300i immunomagnetic automated cell-sorting device.

7.4 Results

7.4.1 Initial TP1 55.3 Production

Utilising a simple culture flask fermentation system the concentration of this antibody in filtered supernatant was measured by murine IgG ELISA (Chapter 2.8) at 1200ng/ml. Unfortunately the hybridoma supernatant from TP1 55.3 proved to be persistently positive (in culture-based assays) for *mycoplasma pneumoniae* despite eradication steps at the Therapeutic Antibody Centre, Oxford and also at the Centre for Applied Microbiology and Research, Porton Down. Although the *mycoplasma pneumoniae* infection could be eradicated from the TP1 55.3 hybridoma cells by exposure to clarithromycin, hybridoma cells retained no ability to secrete functional anti-human CD69 after this treatment.

7.4.2 Bulk Allostimulation Strategies

Upregulation of CD69 on HLA-mismatched $CD3^+$ responder cells after 72 hours) was not significantly different to that seen in 96 round-bottomed-well plates when MLR cultures were in 24 flat-bottomed-well plates or in Stericell $160cm^3$ cell culture bags. However HLA-mismatched responder proliferation after 120 hours of allostimulation was significantly lower when allostimulation was in Lifecell $160cm^3$ cell culture bags compared to that seen after allostimulation in 96 round-bottomed-well plates, (Figure 7.2).
Similarly proliferation at 120 hours (expressed as the percentage RRI) of HLA-matched responders after cytokine-modified allostimulation was lower after larger scale allostimulation than that seen after allostimulation in 96 round-bottomed-well plates (but not significantly so, data not shown).

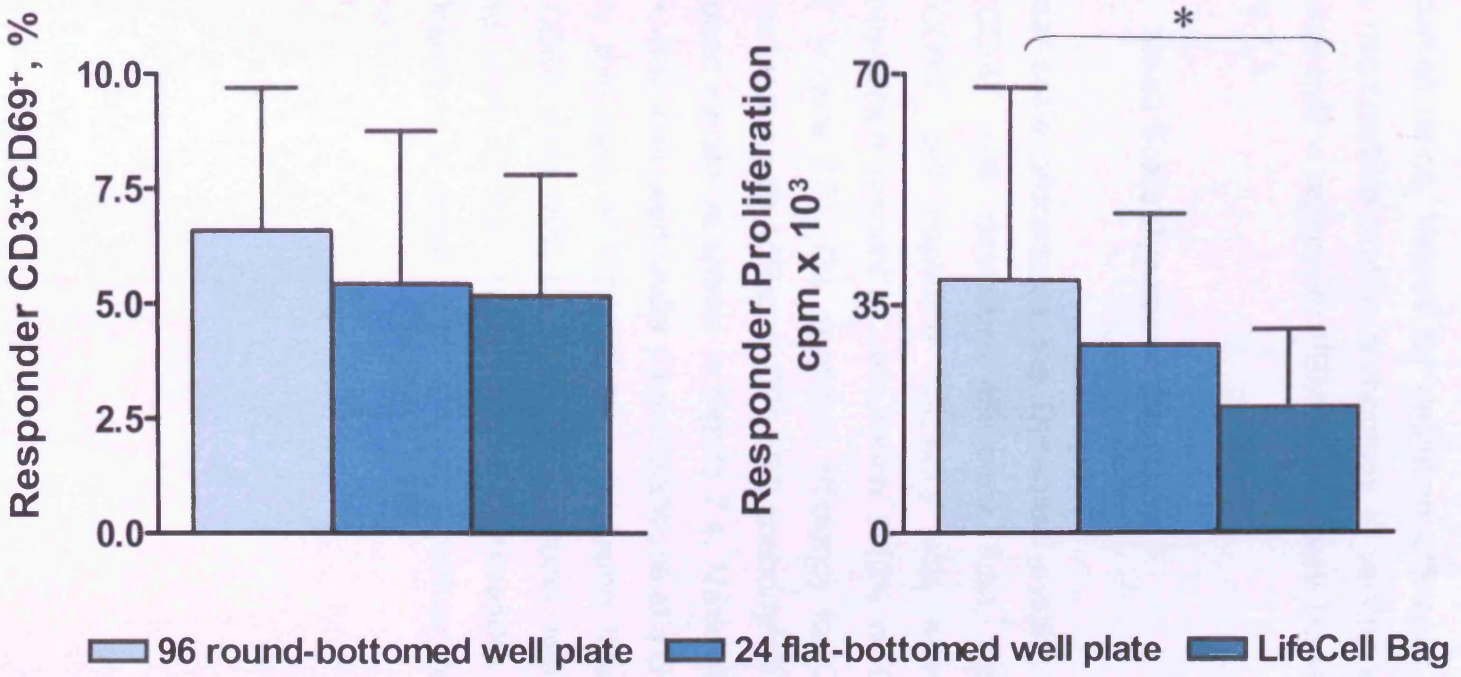

Figure 7.2 HLA-mismatched allostimulation under various different conditions. CD69 expression on $CD3^+$ responders (above that seen with autologous stimulators) after 72 hours of allostimulation is shown on the left and responder proliferation after 120 hours of allostimulation is shown on the right, after co-culture in 96 round-bottomed well plates, 24 well plates or $160cm^3$ LifeCell bags (as detailed in Table 7.2). The results of 5 HLA-mismatched pairs are shown. Error bars represent standard deviation. (*= p</=0.05 in a two-tailed, paired Student's t-test).

7.4.3 Small Scale Eligix Microparticle Depletion

The Eligix system was found to have moderate (and variable) depletion efficiency of $CD3^+CD69^+$ cells (between 40 and 65%) when used at various particle/cell ratios. Values for depletion efficiency of $CD3^+CD69^+$ cells using Eligix microparticle sorting technology at varying concentrations of TP1 55.3 antibody/ million responder PBMCs (at a particle:cell ratio of 25:1) are shown in Figure 7.3.

7.4.4 Small Scale Dynabead Depletion

In small-scale procedures the Dynabead system was found to have better $CD3^+CD69^+$ cell depletion efficiency than the Eligix system. Optimal $CD3^+CD69^+$ cell depletion efficiency was seen with 150ng TP1 55.3 antibody/million responder cells when 5-10% of $CD3^+$ responder cells were $CD69^+$ (Figure 7.3). The depletion efficiency for $CD3^+CD69^+$ cells at varying bead:cell ratios with 150ng of TP1 55.3 antibody/million responder cells with the Dynabead system is shown in Figure 7.4. Maximal depletion efficiency (mean 97% +/-5%) was seen under these conditions at a Dynabead:cell ratio of 5:1.
Initially the yield of $CD3^+CD69^-$ cells using this system was poor (mean $CD3^+CD69^-$ yield 28% +/- 14%) The protocol was duly modified to include a second wash of the magnet-bound Dynabeads, which increased the yield significantly to a mean of 89% +/- 18% without reducing depletion efficiency (Figure 7.5).

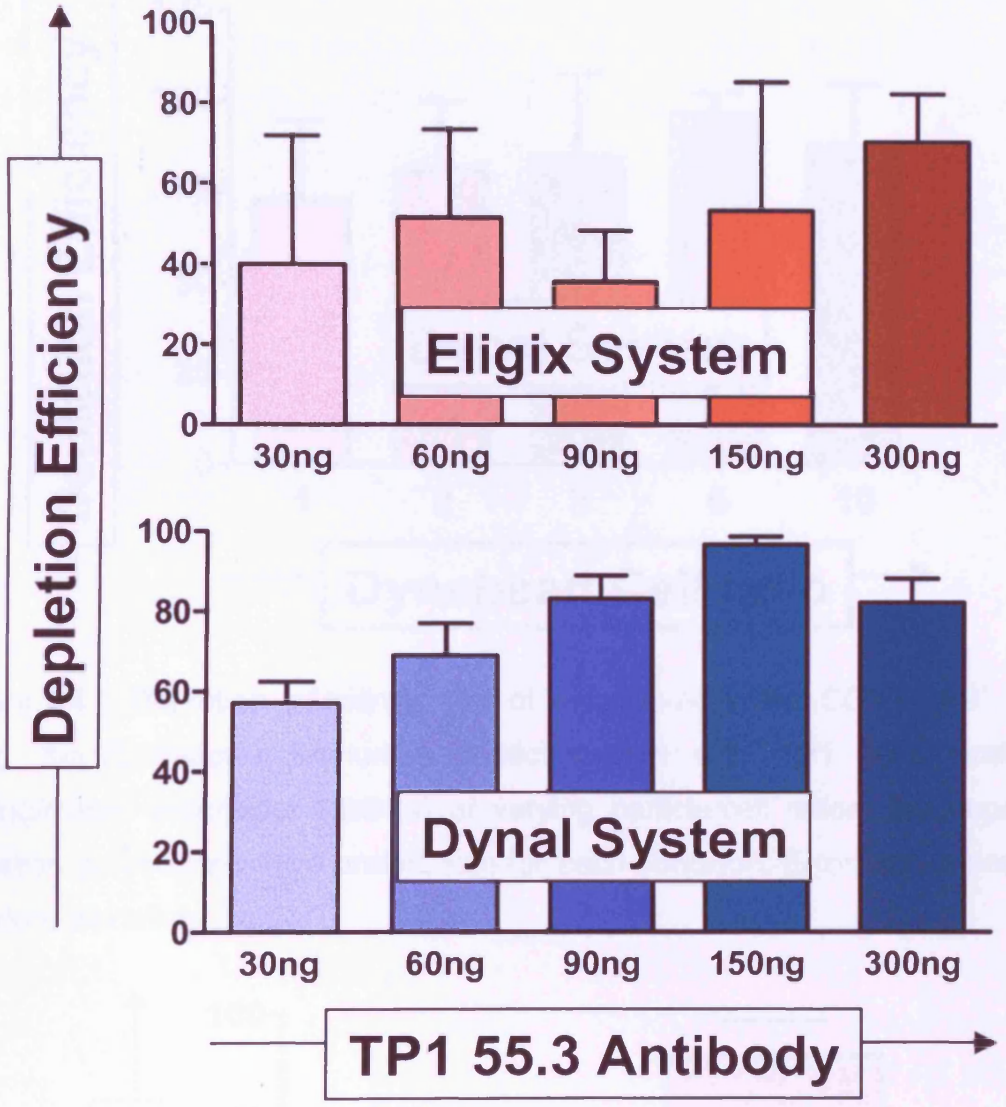

Figure 7.3 Depletion efficiency of mitogen-activated $CD3^+CD69^+$ cells using Eligix microparticle and Dynabead immunomagnetic systems with TP1 55.3 antibody at varying concentrations (per million responder PBMCs). Particle:cell ratio was 25:1 for Eligix depletions and 5:1 for Dynabead depletions. 5-10 separate depletion procedures were undertaken for each condition. Error bars represent standard deviation.

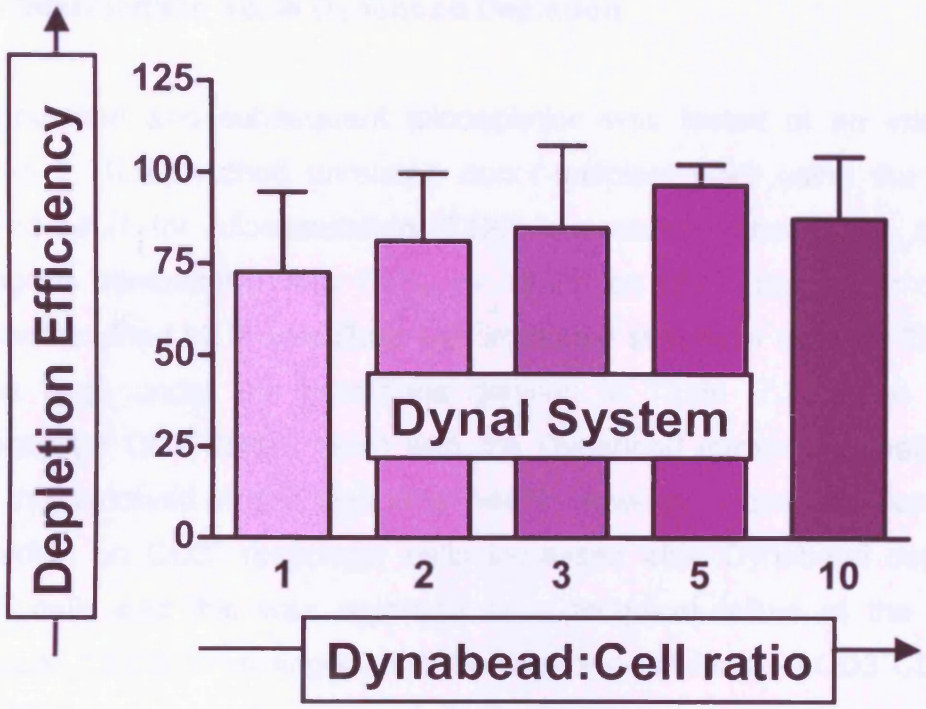

Figure 7.4 Depletion efficiency (%) of mitogen-activated $CD3^+CD69^+$ cells using the Dynabead Immunomagnetic system with TP1 55.3 antibody (150ng/million responder PBSCs) at varying particle:cell ratios. 3-5 separate depletion procedures were undertaken for each condition. Error bars represent standard deviation.

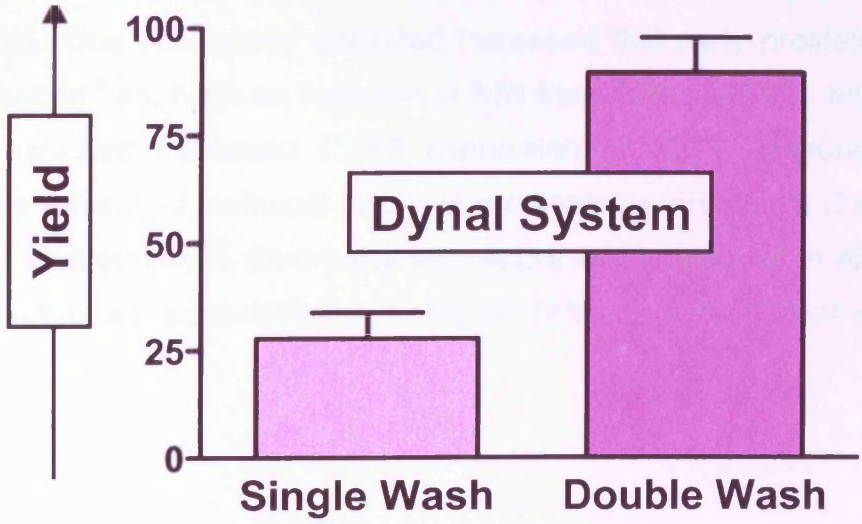

Figure 7.5 Yield of $CD3^+CD69^-$ cells using the Dynabead immunomagnetic system utilising a single or double wash procedure of cells bound to the magnet (TP1 55.3 antibody at 150ng concentration/million responder PBSCs, particle:cell ratio of 5:1). Error bars represent standard deviation.

7.4.5 Intermediate Scale Dynabead Depletion

Allostimulation and subsequent allodepletion was tested at an intermediate scale in 5 HLA-matched unrelated donor-recipient pairs using the cytokine-modified MLR for allostimulation. CD69 expression (above that seen with autologous stimulators) was 7.3% +/- 13.7% on $CD3^+$ responder cells after cytokine-modified MLR co-culture with irradiated stimulator cells for 72 hours in Lifecell bags under the conditions detailed in Table .7.2. Mean depletion efficiency (of $CD3^+CD69^+$ cells) with the Dynabead immunomagnetic system using the handheld MPC-1 was 74%+/-48%. However in one pair (pair 1) CD69 expression on $CD3^+$ responder cells increased after Dynabead depletion of $CD69^+$ cells and this was regarded as a technical failure of the depletion procedure. Of the remaining 4 pairs the depletion efficiency of $CD3^+CD69^+$ cells was 93%+/-14%.

4 of the 5 pairs tested had a first party proliferative response at 120 hours of co-culture in the primary cytokine modified MLR greater than 5% of that seen with HLA-mismatched third party stimulators (RRI>5%).

When allostimulated and CD69-depleted cells were used as responders, proliferation to HLA-matched (first party) stimulators in the secondary cytokine-modified MLR was reduced in 3 of these 4 pairs, 2 to a RRI < 5% and one to a RRI of 5.8%. One pair tested exhibited increased first party proliferation with depleted responders, (with an increase in RRI from 12% to 22%) - this was the pair that exhibited increased CD69 expression on $CD3^+$ responders after depletion as a result of technical failure of the depletion procedure (Figure 7.6). Responder proliferation to third party stimulators was preserved in all samples tested with a mean post-depletion value of 74% +/- 44% of that seen pre-depletion.

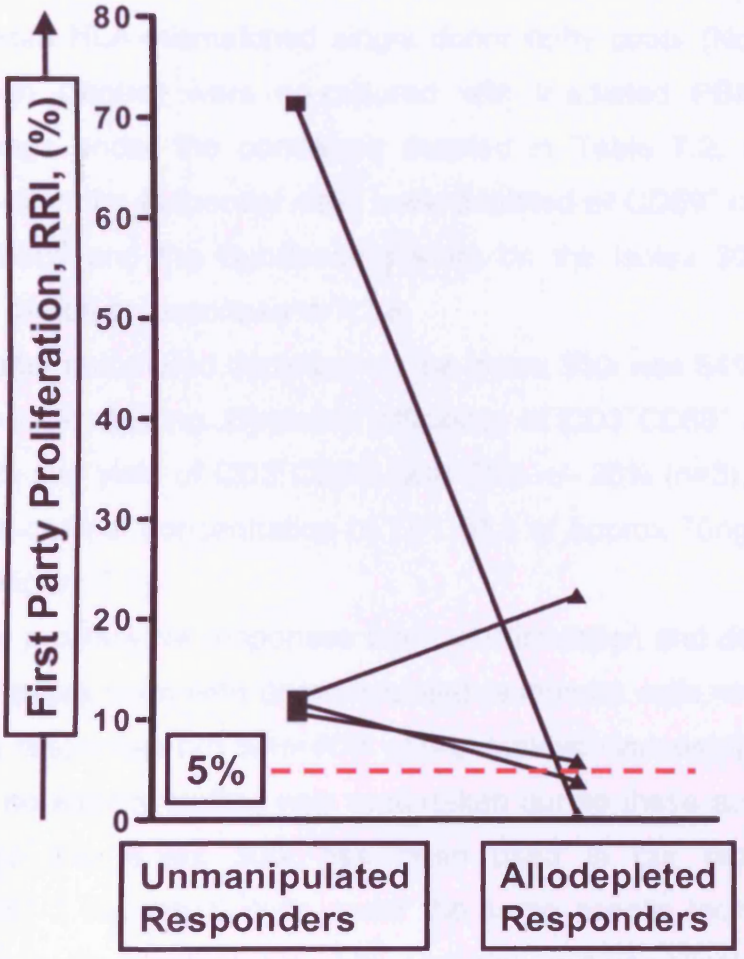

Figure 7.6 Proliferative responses to first party stimulation pre- and post-allodepletion with Dynabeads and CH11 antibody in 4 HLA-matched unrelated pairs utilizing the cytokine-modified MLR. The RRI of 5% is marked; A RRI in the cytokine-modified MLR below this level was associated with a high negative predictive value for acute GvHD in HLA-matched sibling AHSCT.[Bishara, Brautbar, Nagler, Slavin, Leshem, Cohen, and Kedar 1994]

7.4.6 Clinical Scale Dynabead Depletion

PBMCs from HLA-mismatched single donor buffy coats (North London Blood Transfusion Centre) were co-cultured with irradiated PBMC stimulators in Lifecell bags under the conditions detailed in Table 7.2. After 72 hours of allostimulation the responder cells were depleted of $CD69^+$ cells using the TP1 55.3 antibody and the Dynabead system on the Isolex 300i automated cell selection device as described in 7.3.8.

Viability after automated depletion on the Isolex 300i was 84%+/-23% assessed by trypan blue staining. Depletion efficiency of $CD3^+CD69^+$ cells was 93% +/- 10% (n=3) and yield of $CD3^+CD69^-$ cells 75% +/- 28% (n=3), despite having to use a sub-optimal concentration of TP1 55.3 of approx 70ng/million responder PBMCs (Figure 7.6).

First party proliferative responses after allostimulation and depletion were 19% +/- 7% of those seen with unmanipulated responder cells, with preservation of third party responses (95 %+/- 40% of pre-depletion values), (Figure 7.7).

Although no sterility testing was undertaken during these automated depletion procedures the Isolex 300i has been used in our laboratory to select $CD4^+CD25^+$ T regulatory cells under the same aseptic technique utilising the same disposable plastic tubing kits and end-product collection bags as those used to perform a CD69 depletion. Culture samples of the end product of 5 such selection procedures sent for microbiological culture (Department of Microbiology, Royal Free Hospital, London UK) did not demonstrate any evidence of bacterial contamination of the end product thus validating the sterility of the system.

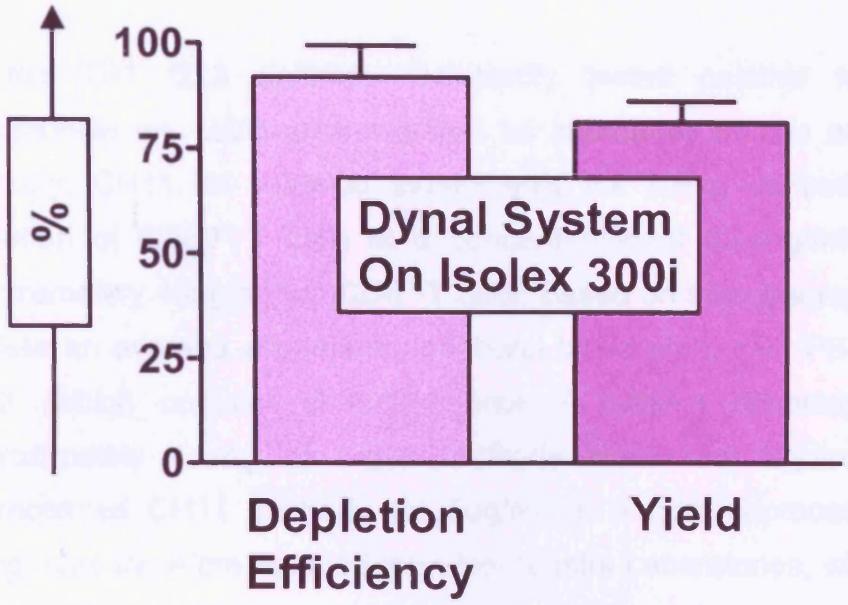

Figure 7.7 Depletion efficiency (% $CD3^+CD69^+$) and yield of viable $CD3^+$ $CD69^-$ cells using the TP1 55.3 antibody, the Dynabead system and the Isolex 300i at a clinical scale (Error bars represent standard deviation, n=3)

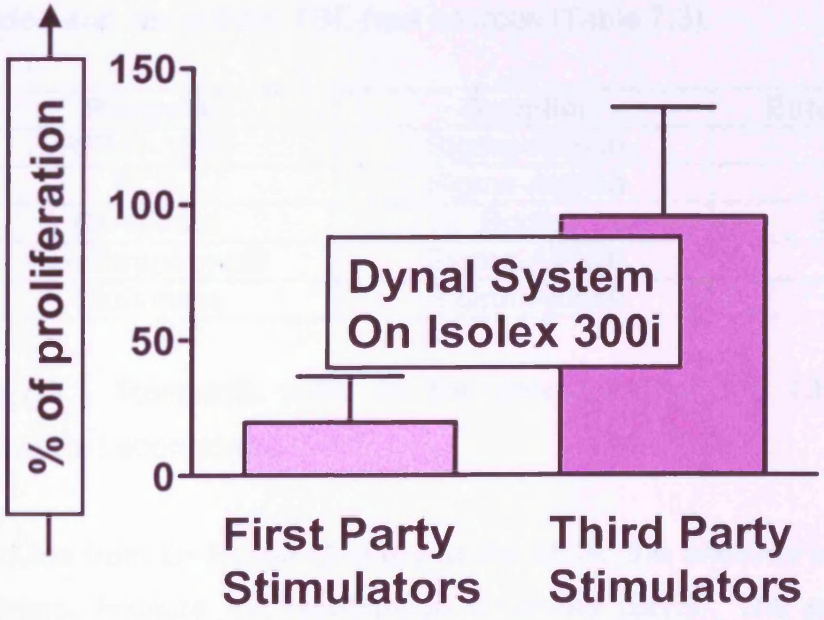

Figure 7.8 Responder cell proliferation after selective allodepletion (using the TP1 55.3 antibody, the Dynabead system and the Isolex 300i at a clinical scale), to first and third party HLA-mismatched stimulators, expressed as percentage of values seen with unmanipulated responder cells. (Error bars represent standard deviation, n=3)

7.4.7 Purification of CH11 antibody

As the TP1 55.3 antibody repeatedly tested positive for *mycoplasma pneumoniae* we sourced and tested an alternative murine anti-human-CD69 antibody, CH11. In scale-up experiments the CH11 antibody gave optimal depletion of $CD69^+$ T cells at a concentration of 62.5ng/million PBMCs i.e approximately 40ng/million $CD3^+$ T cells. Based on this observation, in order to deplete an average allogeneic peripheral blood stem cell (PBSC) graft for an adult (which contains $2\text{-}3x10^8$ donor T cells/kg recipient body weight) approximately 1 mg of CH11 antibody would be required. 4000mls of unprocessed CH11 antibody (at 5ug/ml i.e a total unprocessed amount of 20mg) was therefore sourced from Novocastra Laboratories, who produced the antibody in house from their own hybridoma (using recombinant antigen). Details of reagents used in the production of the CH11 antibody are listed in Table 7.3. The unprocessed CH11 antibody had not been tested for *mycoplasma pneumoniae* (although all reagents were screened). All culture media used at Novocastra Laboratories in production of the CH11 antibody was recorded and came from TSE-free sources (Table 7.3).

Reagent	Supplier	Batch Number
RPM1-1640	Sigma-Aldrich	R8758
FCS	Sigma-Aldrich	F4135
Condimed	Roche	1088947
Pen/Strep/AmpB	Sigma-Aldrich	G7513
Glutamine	Sigma-Aldrich	G7513

Table 7.3 Reagents used in the production of the CH11 antibody at Novocastra Laboratories.

On advice from Dr Elaine Godfrey at the MCA, the antibody was processed at the Bristol Institute for Transfusion Sciences (BITS). The processing of the CH11 antibody is outlined in Figure 7.8. The projected net yield was 47% (i.e 9.4mg of processed CH11 antibody would be produced from 20mg of unprocessed Ch11 antibody). Unfortunately technical problems with the Protein G chromatography column led to retention of most of the CH11 antibody within the matrix of the column (which was not recoverable) and the final yield was less than 1mg of processed antibody.

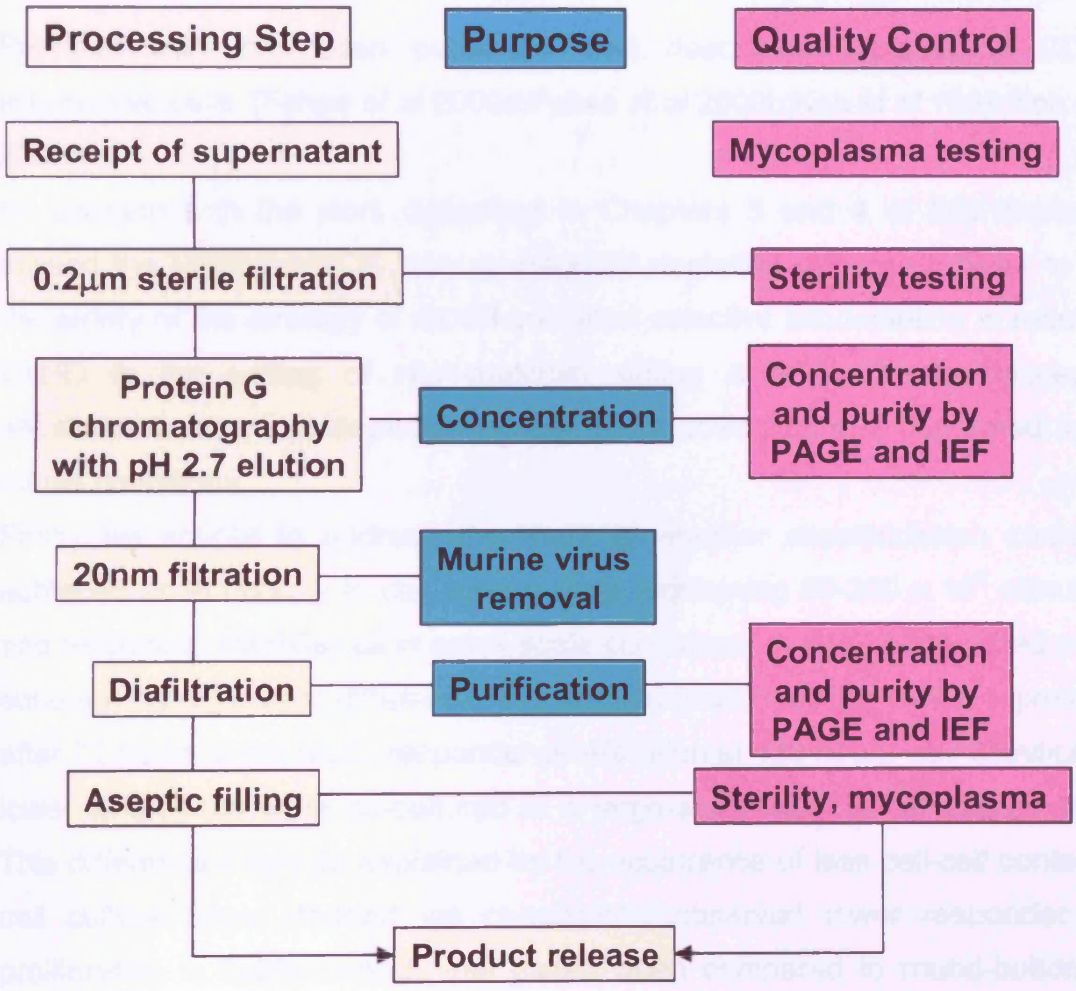

Figure 7.9 The purification process employed and the testing points to ensure quality control for the processing of CH11 anti-CD69 antibody suitable for use *ex vivo* in a clinical study of CD69-mediated allodepletion. PAGE-Polyacrylamide Agarose Gel Electrophoresis, IEF=Immunoelectrofixation

7.5 Chapter Discussion

Previous work has been published work describing depletion of $CD69^+$ alloreactive cells. [Fehse *et al* 2000a;Fehse *et al* 2000b;Koh *et al* 1999;Koh *et al* 2002]

In common with the work described in Chapters 3 and 4 of this thesis, all utilised the Miltenyi/MACS immunomagnetic depletion system. In order to test the safety of the strategy of CD69-mediated selective allodepletion in reducing GvHD in the setting of HLA-matched sibling AHSCT, the techniques of allostimulation and allodepletion needed to be scaled up and performed under sterile conditions.

Firstly we sought to address the issue of whether allostimulation could be achieved as effectively in cell culture bags (containing 50-200 x 10^6 stimulator and responder PBMCs) as in small-scale conditions. In HLA-mismatched pairs, although no *significant* difference was seen in responder cell CD69 expression after 72 hours in the MLR, responder proliferation at 120 hours was significantly lower when cells were co-cultured in a large-scale MLR in cell culture bags. This difference might be explained by the occurrence of less cell-cell contact in cell culture bags. (Indeed we consistently observed lower responder cell proliferation in flat-bottomed- well plates when compared to round-bottomed-well plates, presumably for this reason). This problem was addressed by increasing the responder (and stimulator) cell concentration in bulk MLRs performed in cell culture bags. However to preserve responder cell viability responder cell concentrations in large scale MLRs in cell culture bags were limited to 1.5 x 10^6/ml. Less effective reduction in first party proliferative responses following depletion of alloreactive cells was observed in both intermediate scale HLA-matched depletions (using the CH11 antibody) and large-scale HLA-mismatched depletions performed on the Isolex 300i (with the TP1 55.3 antibody) when compared to small-scale depletions using the MACs/Miltenyi system, despite comparable depletion efficiency of $CD3^+CD69^+$ cells. Less efficient allostimulation might be one explanation for this observation.

Two different immunomagnetic depletion systems were tested at a small-scale using the TP1 55.3 anti-CD69 antibody. The Eligix system was found to have variable depletion efficiency and only achieved depletion efficiency consistently

greater than 50% at the highest antibody concentration tested (300ng/million PBMCs). Thus this system would require large amounts of antibody to deplete alloreactive T cells at a clinical scale, and was still relatively inefficient, and therefore was not selected for further experiments.

The Dynabead system was found to have consistent and high depletion efficiency, maximal at a concentration of TP1 55.3 antibody of 150ng/million PBMCs in small-scale experiments and was therefore selected for further investigation at a larger scale. Initially the yield of $CD3^+CD69^-$ cells post depletion was unacceptably low (at 25%) presumably because non-alloreactive cells were retained on the magnet along with $CD3^+CD69^+$ cells. A second wash step was added to remove more of these $CD3^+CD69^-$ cells, which successfully increased the yield of these cells to consistently more than 80%.

In intermediate scale HLA-matched depletions the Dynabead system was tested with the CH11 antibody. When data from the one depletion procedure that failed was excluded, depletion efficiency of $CD3^+CD69^+$ cells was 93%, equivalent to that seen in small-scale experiments using the Dynabead system and the TP1 55.3 antibody.

The Dynabead system also had the advantage of having an available, CE-marked automated device upon which to perform large-scale depletions under sterile closed conditions, the Isolex 300i. This device is an automated cell-sorting device that uses a semi-closed disposable sterile plastics system and has established use in positive selection of $CD34^+$ cells.[Abonour *et al.* 1998;Cornetta *et al.* 1998] The device has also been shown to be very effective in depletion of T cells when $CD34^+$ cell selection is combined with a depletion procedure.[Martin-Henao *et al.* 2001]

Two stage procedures of $CD34^+$ cell selection followed by depletion of malignant B cells or breast cancer cells in processing of PBSCs from patients with lymphoproliferative conditions have also been reported. The depletion step adds an additional 2 log reduction in the frequency of malignant cells.[Dreger *et al.* 2000;Mohr *et al.* 2001;Paulus *et al.* 1997]

Furthermore, when the two clinical scale devices currently available for $CD34^+$ cell selection PBSC apheresis products, (the CliniMACS and the Isolex 300i), were compared directly (by pooling and splitting two PBSC harvests collected on sequential days from 10 patients and processing half of each pooled harvest on each device), the median recovery of colony-forming units was significantly

greater from the Isolex 300i product as was expansion of cells in either erythroid or granulocytic lineage-specific). This was due to a higher proportion of apoptotic cells in the CliniMACS product. Hence the Isolex 300i product contained fewer apoptotic cells and consequently had greater functional capacity in culture and therefore was deemed the most suitable device upon which to perform the clinical-scale depletion procedure.[Watts *et al.* 2002]

Depletion efficiency of $CD3^+CD69^+$ cells using Dynabeads and the TP1 55.3 antibody at a large-scale (200 x 106 responder cells) on the Isolex 300i device compared favourably with published data.

Dynabeads have been used by Luqman et al at a bench level and on the Isolex device to deplete CD69 alloreactive cells following HLA-mismatched allostimulation. They reported a depletion efficiency of $CD69^+$ cells of 72%+/-8% and a yield of $CD3^+CD69^-$ of 77.3% +/- 7% (n=3) although no data on reduction in functional alloreactivity was presented. [Ho *et al.* 2003]

The depletion efficiency of $CD3^+CD69^+$ HLA-mismatched responders on the Isolex 300i was higher than that seen with Dynabeads at a smaller scale for the conc of TP1 55.3 used (60ng/million cells) (93% vs 70% probably because of the increased efficiency of the depletion process on the automated device, which has a secondary magnet. Sufficient numbers of HLA-matched stimulator and responder cells were not available for assessment of large-scale allostimulation and depletion on the Isolex 300i device, although it is likely that in parallel with HLA-mismatched pairs, depletion efficiency using Dynabeads on the automated device would be equivalent to, or better than that seen at a small-scale.

First party proliferative responses after depletion of HLA-mismatched responders at a large-scale on the Isolex 300i were reduced to 19% of those seen with unmanipulated cells despite a depletion efficiency of >90%. The higher residual first party proliferative responses seen after depletion on the Isolex 300i might have resulted from less efficient allostimulation in cell culture bags (*vide supra*) or from sub-optimal depletion efficiency, perhaps as a result of the limitation of the amount of TP1 55.3 antibody set by the relatively low concentration of this antibody following its in house production in a cell culture flask fermentation system, and the constraints set by the maximal depleting antibody volume permitted on the Isolex 300i device.

$210x10^6$ responder PBMCs were depleted on the Isolex 300i, equivalent to approximately $1.5x10^8$ $CD3^+$ cells. This T cell dose is considerably less than that contained in an average allogeneic PBSC graft for a 70kg adult (approximately 10^{10} $CD3^+$ T cells) although greater than the T cell dose needed to cause GvHD after AHSCT (7×10^7 cells for a 70 kg matched sibling recipient, 7×10^6 cells for a 70 kg matched unrelated recipient and as little as 7×10^5 cells for a 70 kg haploidentical recipient).

For the purposes of the clinical pilot study the primary aim would be to demonstrate safety of infusion of the selectively allodepleted T cell fraction to HLA-matched sibling PBSC recipients. Escalating doses of selectively allodepleted T cells will be re-infused. Only at doses of 10^6 selectively depleted T cells/kg will reach the threshold where GvHD might be expected in this setting with a similar number of non-selected T cells re-infused. The scale up experiments performed on the Isolex 300i have thus shown that the device can adequately allodeplete such numbers of donor T cells (in the HLA-mismatched setting).

Obtaining an antibody safe for clinical (albeit *ex-vivo*) use has been exceptionally difficult. Despite several different strategies we have been unable to eradicate the *mycoplasma pneumoniae* infection from the hybridoma cells that produce the TP1 55.3 antibody without adversely affecting the secretion activity of the cells. The vast majority of the supply of an alternative anti-CD69 antibody (CH11) was lost during purification procedure.

After the failure of the processing procedure of CH11 antibody attempts were made to produce larger quantities of TP1 55.3 antibody and to eradicate the *mycoplasma pneumoniae*. Small-scale experiments demonstrated optimal depletion of $CD69^+$ T cells at a concentration of TP1 55.3 of 150ng/million PBMCs, although good depletion was still seen at concentrations of 60-70ng/million PBMCs at a larger scale using the Isolex 300i. Based on this observation, in order to deplete an entire average PBSC graft for an adult approximately 1-2mg of TP1 55.3 would be needed. Clearly the antibody would need to be produced in larger quantities and processed to ensure both sterility and increased concentration (by 2 logs) in order to be suitable for use at a full clinical scale using the Isolex 300i (which has a maximal volume of depleting antibody of 12 mls).

A fermentation system to produce hybridoma supernatant containing TP1 55.33 antibody at a greater concentration than that produced after cell culture in 80cm3 cell culture flasks (1200ng/ml) has been designed, using cylindrical cell culture flasks and a rotating platform (Jencons, Forest Row, UK).

In order to further concentrate the antibody and to purify it to render it free of infection with *mycoplasma pneumoniae* a process has been designed utilising antibody elution on a Prosep-A chromatography column (Prosep, Elland, UK) followed by pathogen removal on the Intercept Q anion exchanger (Millipore). The latter device efficiently and economically removes picogram to nanogram concentrations of negatively-charged trace impurities resulting in a 5 log reduction in virus and a 3-4 log reduction in pathogen DNA and endotoxin. This procedure has a predicted yield of 85% of TP1 55.3 antibody and will produce a final concentration of antibody of approximately 150 μg/ml. Thus the 1-2 mg of antibody needed to deplete an entire adult PBSC on the Isolex 300i would be contained in a volume of approximately 10mls, within the depleting antibody volume limit stipulated.

Chapter 8 General Discussion

8.1 Allostimulation Prior to Selective Allodepletion

Although non-selective T cell depletion in AHSCT is the most effective method for prevention of clinically significant GvHD, it is associated with increased rate of disease relapse as a result of the removal of T cells with specific anti-leukaemic activity. Therefore the development of techniques to selectively remove, destroy or anergise the T cell component that mediates GvHD from allogeneic haematopoietic grafts have assumed a high priority in recent years. Gribbin et al induced alloantigen-specific anergy in T cells by complete blockade of B7-mediated co-stimulation. In the MLR this resulted in reduced proliferation and lower HTLp frequencies against the original stimulator cells but not against third-party stimulator cells.[Gribben *et al.* 1996] In addition, Taub et al induced specific T-cell tolerance in human T cells by providing them with an alloantigen-specific TCR signal in the presence of wortmannin.[Taub *et al.* 1997]

However, an important drawback of anergy induction is that reintroduction of IL-2 reverses anergy and production of IL-2 plays a pivotal role in human acute GVHD.[Roy *et al.* 1995]

The work presented in this thesis examines the identification of alloreactive T cells and their subsequent removal from the donor T cell pool based on their expression of the activation marker CD69 after co-culture with irradiated recipient cells.

In the HLA-matched AHSCT setting only mHag disparity exists between donor and recipient and these antigens cannot be routinely typed for at this juncture. Moreover if donor/recipient pairs were to be typed for mhags and donors selected on the basis of minimal mHag disparity then this would result in

1. A vastly reduced acceptable donor pool (if the donor was required to be matched at HLA Class I and II and multiple mHag loci in addition).

2. The potential loss of donor T cell GvL activity mediated by donor T cells with specificity for mHags preferentially expressed on recipient leukaemic

cells. In this situation some degree of mHag disparity between donor and recipient would be desirable.

MinorHags have been shown to have tissue–specific expression but haematopoietic lineage-expression has not as of yet been described.

In order to extend the applicability of the allostimulation and allodepletion protocol to most HLA-matched (sibling and unrelated donor) pairs work presented here examines a technique of activating recipient T cells and their subsequent use as APCells in the allostimulation step. It was envisaged that this strategy would be advantageous when applied in the setting of myeloid malignancies when recipient myeloid APCells and leukaemic cells might express shared antigens that might be presented to alloreactive donor T cells. Removal of such cells might lead to a loss of T cells with leukaemia-specific activity with a concomitant reduction in the donor T cell-mediated GvL effect (Figure 8.1).

Conversely an antigen-presenting system utilising myeloid-derived APCs (such as monocyte-derived dendritic cells) might be advantageous when used to present host alloantigens to the donor T cell pool prior to selective allodepletion in the setting of recipient lymphoid leukaemias, when the use of lymphoid APCs might present antigens to the donor T cell pool that are over-expressed on leukaemic cells.

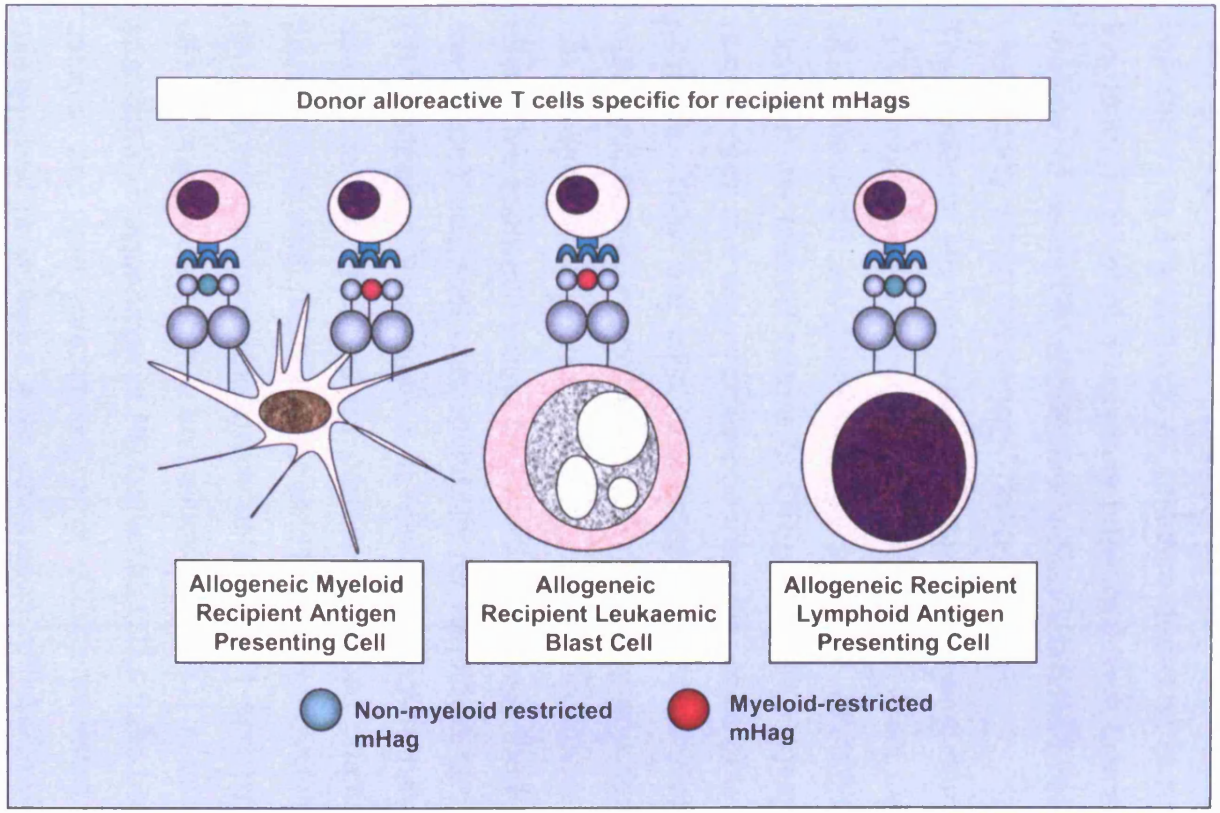

Figure 8.1 The choice of host APCells and the potential for shared expression of lineage-specific mHags on myeloid leukaemic blasts.

8.2 The Retention of Anti-leukaemic Activity after Selective Allodepletion

Although the work presented in this thesis extends previously published work (in both *in vitro* human donor-recipient pairs and a murine model), particularly in HLA-matched setting, the issue of retention of anti-leukaemic activity has not been specifically addressed. Retention of the GvL effect would be of paramount importance to any strategy of selective depletion of host-specific T cells within the donor T cell pool. Previously published work describing the CD25-mediated strategy of selective allodepletion has examined retention of anti-leukaemic blast activity within the donor T cell pool.

The studies of Montagna et al and Mavroudis et al demonstrated that CTLp and HTLp frequencies against recipient leukaemic blast cells were maintained after CD25-mediated allodepletion.[Mavroudis *et al* 1996;Montagna *et al* 1999] However the indirect nature of CTLp and HTLp assays, which involve in vitro restimulation and wide confidence intervals, could potentially obscure significant changes. They are also dependent on the availability of viable recipient leukaemic blasts (in addition to both recipient and donor PBMCs), which were not available for the vast majority of donor-recipient pairs tested herein. An alternative approach would be to indirectly study the frequency of T cells within the donor T cell pool with specificity for candidate tumour antigens such as the PR1 epitope of Proteinase 3. It has been shown that cytotoxic T lymphocytes specific for the PR1 peptide preferentially lyse primary leukaemic blasts from patients with AML,[Molldrem *et al.* 1996a] preferentially inhibit leukaemic CFU-GM colony formation,[Molldrem *et al.* 1997] and have been correlated with clinical responses to IFN-α and allogeneic HSCT in patients with CML.[Molldrem *et al.* 2000] Frequencies of PR1-specific CTLs have been quantified by tetramer assays and are preserved after CD25-mediated allodepletion of HLA-mismatched responders after stimulation with LCLs.[Amrolia *et al* 2003a] This approach might be useful for proof of principle that anti-leukaemic activity in the donor T cell pool is not removed after allodepletion based on CD69 expression but it must be stressed that;

1. HLA Class I tetramer-based assays merely enumerate T cells with high-affinity TCR specificity of the antigen concerned (and give no information

about the functionality of such T cells, or of the presence of $CD4^+$ T cell helper activity).

2. Increased expression of PR1/proteinase 3 on leukaemic blasts is by no means a universal finding in myeloid leukaemias, and when host blast cells are not available (for confirmation of expression of Proteinase 3) then the demonstration of retention of CTLs specific for at least one other myeloid-leukaemia-associated antigen would be desirable. Indeed a pool of tetramers for myeloid-leukaemia specific or-selective antigens could be employed to assess donor T cell residual anti-leukaemic activity, perhaps selected based on individual recipient blast cell phenotype.

Suitable candidate antigens over-expressed on acute myeloid leukaemia blasts include the Wilm's Tumour Antigen (WT-1) and the myeloperoxidase-related antigen MY4. [Scheibenbogen *et al.* 2002] [Braunschweig *et al.* 2000;Molldrem *et al.* 2002]

It is possible that tetramer assays might also be useful in confirming the efficacy and specificity of the selective allodepletion protocol. In HLA-matched donor recipient pairs alloreactive donor T cells will have specificity for mHags and the reduction in frequency of mHag specific T cells within the donor pool could be directly demonstrated by use of mHag-peptide tetramers.

However some mHags are restricted to haematopoietic tissues (e.g. HA-1) and retention of T cells with specificity against such mHags after selective allodepletion might be desirable as such T cells could exert a GvL effect via their host haematopoietic cell-restricted specificity. In order to ensure that such T cells are not lost after selective allodepletion, allostimulation would need to be via non-haematopoietic host cells. Such a protocol has been published describing the use of host keratinocytes as APCs and the subsequent depletion of donor alloreactive T cells by immunomagnetic sorting based on various T cell activation markers. In five HLA-identical patient-donor combinations the effect of depletion of keratinocyte mHag-activated donor T cells was a 10-fold reduction in recipient keratinocyte-HTLp frequency with preservation of recipient leukaemic blast HTLp frequencies.[van Dijk *et al* 1999] However keratinocytes induce anergy in donor T cells (due to the lack of expression of co-stimulatory molecules) and this lack of stimulatory capacity must be corrected by addition of

an anti-CD28 antibody or by transfection of host keratinocytes with B7-1 or B7-2.[van Dijk *et al.* 1996] Additionally the construction of a host keratinocyte cell layer capable of presenting alloantigens on sufficient scale for the clinical application of such a strategy would be a technically difficult proposition.
An alternative approach to demonstrating the retention of donor T cells able to exert a GvL effect would be the use of a murine model. Such a model would need to demonstrate simultaneously the reduction in GvHD associated with the reinfusion of selectively allodepleted T cells and the preservation of an *in vivo* anti-leukaemic effect. Lethally irradiated BALB/c mice might be inoculated with BCL1 cells (a primary leukaemia/lymphoma cell line) and;

1. C57BL/6 T-cell-depleted bone marrow cells. These mice should develop fatal leukaemia.

2. Unmanipulated 5 x 10^6 untreated C57BL/6 T cells. All mice should die of GvHD.

3. Similar numbers of CD69-mediated allodepleted C57BL/6 T cells-recipients should survive more than 100 days without detectable BCL1 tumour cells and free of GVHD.

8.3 The Retention of Anti-viral Activity after Selective Allodepletion

The work presented in Chapter 4 of this thesis demonstrates that the process of allostimulation and subsequent allodepletion (based on the expression of CD69 on alloreactive responder T cells) preserves the majority of functional CMV-specific cells within the donor T cell pool. These are the first data to demonstrate directly the preservation of functional CMV-and EBV- cell frequencies after selective allodepletion of HLA-matched responders and are also the first data to demonstrate this in HLA serotypes other than HLA A*0201. It is of great interest that selective depletion leads to a modest decrease in the frequency of CMV-specific cells using either technique for allostimulation of HLA-matched responders. This would not be of relevance to the clinical application of the technique of CD69-mediated allodepletion as the majority of functional CMV cells are retained. Peggs et al used adoptive transfer of very

small numbers of donor-derived CMV cells after AHSCT which led to massive *in vivo* expansion of $CD8^+$ CMV-specific cells (as detected by HLA A*0201-CMV peptide tetramers). The authors estimated that the infused CMV $tetramer^+$ cytotoxic T-cell doses were about 350–400 T cells/kg recipient body weight and that these were subsequently expanded by 3–5 logs *in vivo*.[Peggs *et al* 2003c] Even if a CMV IgG $^+$ HLA $A*0201^+$ donor with a baseline frequency of $CD3^+CD8^+$ CMV-$Tetramer^+$ cells of 1% lost 30% of these cells during the process of selective allodepletion (based on CD69 expression) in the HLA-matched setting, the selectively allodepleted T cell pool would still contain a frequency of $CD3^+CD8^+$ CMV $tetramer^+$ cells of 0.7%. This would equate to a $CD3^+CD8^+$ CMV $tetramer^+$ cell dose of approximately 2000 cells/kg recipient body weight if as little as 10^6/kg recipient body weight selectively allodepleted T cells were infused, well above the threshold level of CMV-specific T cells shown to expand *in vivo* after adoptive transfer, and confer clinical benefit to the recipient in terms of clearance of CMV viraemia. Additional work on the retention of virus-specific cells after selective allodepletion crucially needs to identify the presence of adequate numbers of pathogen-specific $CD4^+$ cells in allodepleted T cell pool. It remains difficult to quantify the frequency of such cells as HLA Class II tetramers remain technically challenging to construct and ELISpot assays would need to be augmented by an additional antigen-presenting cell system in order for HLA Class II-restricted pathogen antigens to be effectively presented to pathogen-specific $CD4^+$ T cells. One approach for the quantification of CMV-specific $CD4^+$ T cell responses would be to assess CMV whole antigen-stimulated intracellular IFN-γ generation by flow cytometry. This strategy requires additional T cell stimulation with anti-CD28 antibody and has the advantage of not being restricted to a given HLA Class II type (as many different CMV antigens could be processed from the whole CMV antigen source). The pivotal role of $CD4^+$ help in maintaining a functional $CD8^+$ CMV-specific response post AHSCT has recently been identified by Gratama et al and the retention of CMV-specific $CD4^+$ cells after the process of selective allodepletion has yet to be demonstrated. [Cornelissen *et al.* 2003]

8.4 The Retention of T regulatory cells after Selective Allodepletion

The kinetics of expression of two activation antigens (CD69 and CD25) on responder cells after alloantigen expression have been closely examined after both HLA-mismatched and HLA-matched allostimulation. These activation antigens were known to have different kinetics of expression on alloresponding T cells, with CD69 expression occurring earlier than CD25 expression.[Craston *et al* 1997] The co-expression of both antigens on alloresponding T cells has not previously been described in the literature. The results presented in Chapter 5 of this thesis demonstrate that three responder cell subsets can be identified by their expression of one or the other or both of these activation markers although the possibility that individual cells move between these cell subsets over time during the alloresponse cannot be discounted. Thus the strategy of selective allodepletion based on the expression of both of these activation antigens at a single time point, should improve the efficacy of depletion of alloreactive cells and the single report in the literature of this strategy supports this hypothesis.[Fehse *et al* 2000a] Whether sequential depletions of $CD69^+$ alloresponders followed by $CD25^+$ alloresponders improves the efficacy of selective depletion above that seen with a dual depletion process based on the expression of both antigens at a single time point remains to be determined. The strategy of selective allodepletion based on the expression of CD69 alone on alloresponders has the advantage of preservation of both frequency and function of $CD4^+CD25^+$ T regulatory cells within the donor T cell pool, although the significance of this experimental observation remains to be determined as the role of such cells with in the donor T cell pool in human AHSCT remains a subject of great debate.

8.5 The Phenotype of Alloreactive and Non-alloreactive T cells

An experimental technique for the determination of TCR Vβ sub-family distribution on alloreactive and non-alloreactive responder cells following allostimulation in the MLR was described in Chapter 5. This technique, by which $CD69^+$ and $CD69^-$ responder cells can be identified and TCR Vβ sub-family distribution measured by flow cytometry can reliably differentiate between

stimulator cells and responder cells. This is not the case for methods previously used to measure TCR Vβ sub-family spectratype of alloresponder cells in the MLR by DNA PCR for CDR3 length. As expected some skewing of TCR Vβ sub-family distribution was seen in both HLA-mismatched and HLA-matched alloresponders with increases in frequency of several individual TCR Vβ sub-families in each individual tested suggesting responses to several alloantigens in both settings. Moreover the non-alloreactive donor T cell pool maintained a TCR Vβ sub-family distribution very close to that of baseline donor T cells for both $CD4^+$ and $CD8^+$ cells, which would support the hypothesis that the clinical reinfusion of selectively allodepleted T cells contain a broad spectrum of T cell immune activity. A very recent publication where TCR Vβ spectratyping of HLA-mismatched alloresponder cells (identified and sorted by flow cytometry on the basis of the dual expression of $CD4^{HI}$ and $CD38^+$) was measured by PCR for CDR3 has confirmed this observation.[Martins *et al.* 2004]

The memory/effector phenotype analysis of alloresponder T cells (based on the expression of CD45RA isoform and the chemokine receptor CCR7) has demonstrated that after HLA-mismatched allostimulation, $CD4^+CD69^+$ alloresponder T cells contain a mixture of T_{CM}, T_{EM} and naïve T cells, with a modest but statistically significant increase in the proportion of T_{EM} cells compared to that seen in baseline responder T cells. The significance of this observation remains to be determined and suggests that a proportion of human T cells, identifiable as alloresponders by upregulation of expression of CD69, have either previously encountered alloantigen or have shared TCR affinity with alloantigen and previously encountered (pathogen-related) antigen.

No data have been published specifically describing T_{EM}, T_{CM} and naïve subsets based on this classification, although there is an increasing body of evidence showing $CD62L^-$ cells (which contain T_{EM} cells) are unable to act as effector cells mediating GvHD in murine models, and $CD62L^-$ human cells have been shown to be hyporesponsive (in terms of proliferative responses) to alloantigen in *in vitro* experiments.[Anderson *et al.* 2003;Chen *et al.* 2004b;Foster *et al.* 2004b;Xystrakis *et al.* 2004] It may also therefore be the case that $CD4^+CD69^+$ human alloresponder T_{EM} cells, although present in alloreactive cell fractions are not able to mediate GvHD. It would be fruitful to correlate the memory and

effector T cell subsets (as defined by Lanzavecchia and Sallusto) in $CD69^+$ alloreactive cells with their expression of CD62L to further elaborate this.

8.6 The use of CD69 to identify CMV-reactive T cells

CD69 as an activation antigen upregulated with relatively rapid kinetics on the surface of T lymphocytes could be used to identify cells responding to antigenic stimuli other than alloantigens and this possibility was examined with stimulation of donor T cells from HLA $A*0201^+$ CMV IgG^+ individuals by the CMV integument protein pp65-derived NLV nonamer. CMV specific $CD8^+$ cells can be identified by their expression of CD69 following relatively short periods of stimulation without additional cytokine or antibody stimulation. Such responder cells demonstrate some evidence for skewing of TCR Vβ sub-family distribution and the data presented in Chapter 6 are consistent with the limited amount of published data. Data have been published describing the selection of CMV-specific HLA A*0201-NLV Tetramer $^+$ cells by flow cytometry, however this strategy is restricted to HLA $A0201^+$ individuals and the CMV-specific cell fraction would contain only $CD8^+$ cells, with no $CD4^+$ CMV-specific cells that are crucial for maintaining donor-derived CMV specific responses post-AHSCT.[Braunschweig *et al* 2000;Cornelissen *et al* 2003;Keenan *et al* 2001]

It is feasible to imagine the development of a system whereby CD69 expression on T cells is used to identify and to positively select donor T cells with CMV-specificity and this system could be applied to donors with a variety of HLA Class I serotypes. A wide selection of immunogenic CMV-derived peptides with known HLA Class I restriction have been identified and are commercially available and indeed several were used and shown to reliably induce IFN-γ responses in ELISpot assays in the work described herein. However for optimal impact in clinical use such a system would need to utilise a more developed antigen-presenting system, perhaps with the use of whole CMV antigen and enrichment of donor monocyte-derived dendritic cells able to present HLA Class II-restricted CMV antigens to $CD4^+$ T helper cells.

Any strategy to select CMV-specific T cells by antigenic/peptide stimulation of T cells and their subsequent selection based on expression of CD69 might be disadvantaged by the existence of donor T cells with TCRs with shared affinity

for both CMV antigens and alloantigens. Such cells, if used for adoptive immunotherapy, might expand *in vivo* and mediate GvHD. Data presented in Chapter 6 demonstrates that some individuals share preferential TCR Vβ sub-family distribution in both CMV NLV peptide-stimulated $CD69^+$ T cells and HLA-matched allostimulated $CD69^+$ responder T cells supporting the existence of such shared-affinity T cells. Further experiments to confirm this hypothesis could include TCR Vβ spectratype analysis (by CDR3 PCR) and subsequent sequencing to identify accurately T cell clones responding to CMV peptides and to allostimulation. CMV-responders could be sorted by positive selection of $CD69^+$ cells after NLV stimulation whereas PKH-26 labelled responder T cells after allostimulation could be sorted by flow cytometry based on expression of CD69 and PKH-26 dye to accurately distinguish them from stimulator cells in the MLR. Functional experiments could be performed to assess the alloproliferative capacity (as responders in the MLR) of T cells within the CMV-reactive pool after NLV stimulation and compared to that of unmanipulated cells and CMV-peptide non-responders.

In order to minimise the potential for GvHD of CMV-peptide/antigen-stimulated allogeneic T cells a sequential manipulation programme could be employed with allostimulation and subsequent depletion of alloreactive responder cells based on their expression of CD69. The non-alloreactive donor T cell pool could then be stimulated with CMV antigen/peptide and CMV-specific non-alloreactive responder cells positively selected based on their expression of CD69. Using this sequential strategy a pool of viral peptides from different pathogens important in AHSCT (e.g. EBV, RSV, adenovirus) could be used simultaneously to stimulate the donor T cell pool after allostimulation and allodepletion to generate a cocktail of non-alloreactive virus-specific donor T cells for adoptive immunotherapy post AHSCT to improve immune reconstitution without GvHD.

8.7 Testing the CD69-mediated Selective Allodepletion Strategy in a clinical pilot study of AHSCT for AML

Many techniques have been investigated to identify and selectively remove alloreactive T cells from the donor T cell pool to reduce GvHD and improve immune reconstitution. Many of these involve cell sorting by flow cytometry, a technique difficult to develop to a clinical scale whilst maintaining cell viability and sterility. The identification of alloreactive T cells within the donor T cell pool by their expression of CD69 has several distinct advantages over many such methods. These include more rapid expression after allostimulation than other activation antigens and the absence of any T cell subsets with constitutional expression of CD69 that would be removed as bystanders during allodepletion based on CD69 expression. Antibody-mediated cell selection procedures are potentially more specific than the use of immunotoxins, which can exert a non-specific toxic effect mediated by their ricin moiety. Moreover CD69-mediated allodepletion led to virtual abrogation of GvHD in a totally MHC mismatched murine model and was associated with a significant survival advantage of recipients of non-allodepleted T cells. The major disadvantage of the CD69-based strategy is the lack of an available clinical-grade anti-CD69 antibody. The scale-up experiments presented in Chapter 7 demonstrate the efficacy of two potential anti-CD69 antibodies and highlight some of the problems encountered in the process of purification and production of such antibodies.

We have designed a clinical pilot study to test the safety of the CD69 allodepletion strategy in adult AHSCT recipients. Adult patients with acute myeloid leukaemia will be entered into the study. They will receive allogeneic PBSC grafts from HLA-matched sibling donors after chemotherapy, immune suppression and fractionated total body irradiation. Recipients will be conditioned with Fludarabine ($30mg/m^2$) on days –9 to –7 by intravenous infusion and with cyclophosphamide 60 mg/m^2 on days –6 and –5 by intravenous infusion. Total body irradiation will be given in 8 fractions over 4 days on days –4 to-1 to a total dose of 1440cGy. The reinfusion of HLA-matched sibling donor PBSC will be split into two fractions, given at different times. The donor PBSC will be undergo positive selection for $CD34^+$ cells under sterile conditions using the Isolex 300i. The $CD34^+$ fraction will be reinfused at day 0 to ensure rapid neutrophil and platelet engraftment.

The $CD34^-$ T cell fraction will then be cultured in cell culture bags with cytokine pre-stimulated irradiated recipient mononuclear cells to stimulate alloreactive T cells within the donor T cell pool. The $CD69^+$ alloreactive donor T cells will then be depleted with anti-CD69 antibody on the Isolex 300i and the $CD69^-$ non-alloreactive donor T cell pool will be cryopreserved. The allodepleted fraction will be tested for efficacy and specificity of allodepletion in a secondary cytokine-modified MLR with first and third party stimulators, and for microbiological infection Fractions found to have residual alloreactivity or to be bacterially contaminated will be discarded. Allodepleted T cells with a post-depletion RRI of 5% or less (as defined by Bishara et al,[Bishara *et al* 1994]) will be reinfused to patients at Day +12 to aid immune reconstitution, initially at a dose of 10^5 T cells/kg recipient body weight. If no severe GvHD is seen in the first cohort of patients, the next 5 will receive a dose of $CD69^-$ allodepleted T cells of $3x10^5$/kg recipient body weight and if no severe GvHD is seen in the second cohort a further 5 patients will receive a dose of $CD69^-$ allodepleted T cells of 10^6/kg recipient body weight. A control group of patients will receive an identical CD34-selected transplant with no allodepleted T cell reinfusion. Detailed SOPs for the validation and execution of all steps involved in the process of production, testing and release of selectively allodepleted T cells have been produced and are currently being tested pending the production of sufficient quantities of clinical-grade anti-CD69 antibody.

Only when tested in such a clinical setting will the safety of the strategy of re-infusing CD69-mediated selectively allodepleted donor T cells be ascertained and the efficacy of this strategy confirmed in reducing and/or preventing clinically significant GvHD. The donor T cell alloresponse *in vivo* is far more complex than any *in vitro* model that has been developed to quantify the alloresponse (such as proliferation assays or cytokine release assays) and thus the ultimate realisation of the technique will be its use *in vivo*.

The speed and breadth of immune reconstitution after the infusion of selectively allodepleted T cells using this strategy will be monitored quantitatively and qualitatively over time with flow cytometric enumeration of lymphocyte subsets ($CD3^+CD4^+$, $CD3^+CD8^+$, $CD3^-CD56^+$, $CD4^+CD25^+$ etc) and TCR Vβ CDR3 spectratyping and TCR Vβ sub-family frequency distribution in $CD4^+$ and $CD8^+$ subsets by the flow cytometric technique validated in Chapter 5. Naïve and

memory T cell subsets will also be enumerated based on their expression of CD45RA and CCR7 over time post AHSCT.

The ability of selectively allodepleted donor T cells to provide donor-derived immunity against specific pathogens will be monitored over time by the measurement of frequencies of viral-specific T cells within recipient blood by CMV-HLA Class I tetramer and CMV and EBV ELISpot assays.

8.8 Other Strategies for Potentiating the GvL Effect

In addition to reducing GvHD whilst augmenting immune reconstitution, the other potential advantage of the strategy of reinfusion of selectively allodepleted donor T cells is preservation of the GvL effect, which is known to be lost after profound non-selective T cell depletion. Other strategies for potentiating the GvL effect without causing GvHD have been explored in recent years.

One such strategy is the amplification of GvL-reacting lymphocytes *in vitro.*

The precursor frequency of allogeneic leukaemic blast-reactive T cells is very low, (typically in the order of 1/1,000,000 in the unprimed donor).[Jiang *et al.* 1991]

Attempts to expand T cell clones with leukaemic blast -specificity would therefore require very efficient *in vitro* T cell selection and expansion techniques. Current techniques to raise donor-specific anti-leukaemic blast cell T cell lines for clinical use are inconsistent - not all donor-recipient pairs generate specific cytotoxic anti-leukaemia clones, and T cell expansion *in vitro* is unreliable.[Falkenburg *et al.* 1993] This may be due in part to the failure of the leukaemic blast cell stimulators to present an immunodominant antigen to the responder. The current inability to identify tissue-restricted antigens on leukaemic blasts is a great limitation to generating anti-leukaemic T cell clones. In this regard the generation of anti-leukaemic cytotoxic T cells responding to peptides derived from the myeloid-restricted granule protein proteinase-3 may serve as a model for generating strong T cell responses to identified antigens.[Molldrem *et al.* 1996b] We are in the process of developing an *in vitro* strategy of selective allodepletion (based on CD69 expression on alloresponder cells) followed donor T cell stimulation by anti-CD28/IL12-stimulated AML blast cells to raise non-alloreactive AML-specific T cells for subsequent *in vitro* expansion, with potential for clinical use in relapsed AML patients.

An alternative approach to potentiate donor anti-tumour T cell activity is the use of vaccines based on tumour antigens, which could be administered to the donor, or the recipient, after transplantation to boost specific immunity. Some progress has been made in the immunotherapy of multiple myeloma by vaccinating the donor with myeloma protein to generate idiotype-specific T cells.[Kwak *et al.* 1996;Kwak *et al.* 2004] However, it has yet to be demonstrated that donor vaccination improves the already demonstrable graft-versus-myeloma effect after allogeneic BMT.[Alyea *et al.* 2001]

Other possible immunogenic leukaemic blast cell antigens suitable for use as vaccines are peptides derived from leukaemia-specific fusion proteins (such as the bcr-abl products p210 or p190 in CML or ALL, and the t(15;17) fusion protein in acute promyelocytic leukaemia. Preliminary results from a Phase 2 clinical trial have demonstrated safety of administration of a tumour-specific, bcr-abl-derived fusion peptide vaccine to patients with CML, and also that such a vaccine elicits a bcr-abl peptide-specific T-cell immune response, although the results are too early to show any clinical benefit of such a strategy.[Cathcart *et al.* 2004] However attempts to generate donor-derived T cells specific to the t(15;17) fusion protein for the adoptive treatment of acute promyelocytic leukaemia has proved less successful.[Dermime *et al.* 1996]

As more is learned about the defects in surface expression of critical molecules on malignant cells, it may become easier to design ways to correct defects in antigen-presentation and co-stimulation associated with leukaemic blasts and render them more susceptible to immune attack. IFN-γ and GM-CSF upregulate MHC molecule expression and render defective leukaemic blast cells susceptible to cytolysis by allogeneic CTLs.[Dermime *et al.* 1997]

The importance of co-stimulation by the leukaemia cell in generating a strong immune response has opened up the possibility of using leukaemia cell vaccines transfected with the B7 co-stimulatory molecules.[Schultze *et al.* 1996] In animal models this can render nonimmunogenic tumours strongly immunogenic. Other promising strategies include transfection of AML blasts with co-stimulatory molecules and the IL-12 gene to improve antigen-presentation to autologous or donor T cells.[Dunussi-Joannopoulos *et al.* 1999]

Recently the role ability of allogeneic donor NK cells in providing a sustained anti-leukaemic effect has been identified. One of the functions of HLA Class I alleles is interaction with NK cells. Receptors termed killer immunoglobulin-like

receptors (KIRs) on NK cells recognize HLA Class I molecules. KIR2DL1 (CD158a) recognises HLA C alleles belonging to the C2 group and KIR2DL2 and KIR2DL3 (CD158b) recognises HLA C alleles belonging to the C1 group. KIR3DL1 recognises HLA Bw4 alleles. Interaction between donor KIR and recipient HLA Class I allele inhibits reactivity of the NK cell. Failure to recognize the appropriate KIR ligand on a mismatched cell can trigger NK cell elimination of that target cell. Recent analysis of haploidentical haematopoietic transplantation for acute myeloid leukaemia has shown a reduction of graft failure, GvHD and relapse in those with KIR ligand incompatibility in the graft-versus-host direction.[Ruggeri *et al.* 1999a] Furthermore murine models have demonstrated the ability of KIR mismatched allogeneic NK cells to exert an anti-leukaemic effect without causing GvHD.[Ruggeri *et al.* 2002] The anti-leukaemic effect of allogeneic KIR mismatched donor NK cells is limited to myeloid leukaemic blasts that express LFA-1, necessary for NK-mediated cytotoxicity. The effect of KIR mismatch is most prominent in the haploidentical setting where the graft has been profoundly depleted of T cells. The role of donor KIR mismatch is less well established in mismatched unrelated donor AHST. Davies et al reported a series of 175 such AHSCTs and found no effect of KIR mismatch (assessed by HLA-Bw4 and HLA-C alleles as described by Ruggeri et al,[Ruggeri *et al.* 1999b]) on relapse or survival, although only a small proportion of recipients had AML.[Davies *et al.* 2002] However Giebel et al have reported 130 unrelated donor AHSCTs with pre-transplantation antithymocyte globulin (ATG) as part of recipient conditioning. Recipients with KIR ligand-incompatible donors had a higher probability of overall survival and disease-free survival and lower relapse rates compared with those without KIR ligand incompatibility. All patients with myeloid malignancies receiving transplants from KIR ligand–disparate donors were alive and disease-free with a median follow up of 4.5 years.[Giebel *et al.* 2003] These data support the association of NK cell alloreactivity with better outcome after unrelated donor AHSCT when ATG is used as part of GvHD prophylaxis. The relative importance of KIR mismatching in AHSCT may therefore depend on the level of T cells within the graft. Additionally it is becoming increasingly apparent that donor NK cells express activatory receptors (for which the ligands remain unidentified) as well as inhibitory receptors. In HLA A, B and DR-matched sibling AHSCT performed for myeloid disease, overall survival was worse in patients homozygous for group 2

HLA-C than in patients who carried a group 1 HLA-C (C1) allele. This effect was seen only when the donor additionally carried the activating KIR gene KIR2DS2.[Cook *et al.* 2004]

Moreover it has been shown that KIR expression within individuals is heterogeneous and independent of anticipated HLA class I ligands.[Becker *et al.* 2003] Thus it seems that the role of donor allogeneic NK cells may be an important one in exerting a GvL effect after AHSCT but this may not simply rely on the predictable KIR mismatch associated with HLA C group disparity between donor and recipient. Additionally, the beneficial effect of donor NK cell alloreactivity is masked by the effects of residual alloreactive donor T cells (even after non-selective T cell depletion). [Bishara *et al.* 2004;Lowe *et al.* 2003] In the HLA-mismatched setting, selective depletion of alloreactive T cells using the CD69-mediated strategy might provide the beneficial effects of donor T cells (GvL, immune reconstitution) without GvHD thus permitting the beneficial effects of KIR mismatched donor NK cells to impact on recipient survival.

8.9 Final concluding remarks

The strategy of selective allodepletion of HLA-matched donor T cells based on removal of alloreactive cells expressing the activation antigen CD69 has been examined *in vitro*.

Donor-derived T cells are now recognised as major determinants of outcome post AHSCT by providing a GvL effect and host immune reconstitution. The work presented in this thesis described modification of the host antigen presenting system that enables the strategy to be applied in both HLA-matched unrelated donor-recipient pairs and HLA-matched sibling donor-recipient pairs. The strategy retains the majority of functional anti-CMV and anti-EBV T cells within the donor T cell pool. The alloreactive and non-alloreactive T cell subset phenotype and TCR Vβ sub-family distribution has been described. CD69 may be used to identify donor T cells specific for CMV and thus the system could be used in sequential allostimulation and viral antigen stimulation to provide non-alloreactive pathogen-specific donor T cells for safe adoptive T cell therapy to enhance recipient immune reconstitution post AHSCT.

Ultimately, the true test of safety and efficacy of the CD69 selective allodepletion strategy is in the setting of a clinical pilot study and the

development and testing of cell selection systems suitable for the production of selectively allodepleted donor T cells for such clinical use have been described.

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